EXTRACELLULAR COMPLEX PEPTIDO-POLYSACCHARIDES OF PENICILLIUM FELLUTANUM (I) EXTRACELLULAR RESERVE OF INTRACELLULAR CHOLINE DERIVATIVES (II) ITS INVOLVEMENT IN OSMOREGULATION

By

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Abstract of Thesis Presented to the Graduate School
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EXTRACELLULAR COMPLEX PEPTIDO-POLYSACCHARIDES OF PENICILLIUM FELLUTANUM: (I) EXTRACELLULAR RESERVE OF INTRACELLULAR CHOLINE DERIVATIVES, (II) ITS INVOLVEMENT IN OSMOREGULATION

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Penicillium fellutanum (formerly *P. charlesii* G. Smith) produces an extracellular complex polysaccharide, peptidophosphogalactomannan (pPGM). It is composed of mannosyl, galactofuranosyl, amino acyl and a variable quantity of phosphodiester residues such as those of choline and ethanolamine. The major objective of this study was to determine the physiological role(s) of the α-D-mannosyl-phosphocholine residues in this polymer.

Mycelia grown in low phosphate (2 mM) medium contains glycine betaine and 1.5-fold more choline-O-sulfate than those grown in high phosphate (20 mM) medium. These choline derivatives originate from the phosphocholine in extracellular pPGM, suggesting that pPGM is an extracellular reserve of choline in *P. fellutanum*. The high levels of nonspecific phosphocholine diester phosphocholine hydrolase (PC:PCH) and acid

phosphomonoesterase observed in the low phosphate culture medium are likely related to the release of phosphocholine from pPGM and hydrolysis of phosphocholine, respectively

Under sulfate sufficient conditions the level of choline-O-sulfate increases with decreased level of glycine betaine and *vice versa* under sulfate deficient conditions. Free choline was not detected in a significant level in the mycelia tested The results suggest that glycine betaine may be another endogenous reserve of choline in *P. fellutanum* under low phosphate conditions.

Penicillium fellutanum grows in medium containing 3 M NaCl, indicating that it is an osmotolerant and xerotolerant fungus The osmoregulatory compatible solutes in this fungus are glycerol, glycine betaine, choline-O-sulfate Exogenous choline or sulfate levels enhance osmotolerance, indicating that both are indirect osmoprotectants in this fungus

The mycelia grown in low phosphate medium with 2 M or greater amounts of NaCl showed increased production of PC PCH, compared with those mycelia stressed with 0 5 or 1 0 M NaCl The ^{31}P-NMR spectra of the pPGMs isolated from the low phosphate cultures grown in 3 M NaCl showed that more phosphocholine is released during salt stress than in unstressed cultures Accumulation of glycine betaine and choline-O-sulfate becomes more significant with over 1 M NaCl stress These results show that phosphocholine in extracellular pPGM and PC PCH activity are involved in osmoregulation in *P. fellutanum* grown under low phosphate and high osmotic stress conditions.

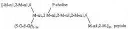
CHAPTER 1
INTRODUCTION

Filamentous fungi produce extracellular complex peptido-polysaccharides (Gander, 1974) The structural chemistry of fungal polysaccharides has been extensively reviewed by Gander (1974), Gorin (1981), and Barreto-Bergter and Gorin (1983) However, their roles in fungal physiology other than as structural components of cell wall or as reserve sources of nutrients (Bull, 1972, Kohama et al , 1974 Tuekam and Gander, 1993) are largely unknown.

The early research on these fungal polymers in *Penicillium charlesii* (recently renamed, *P fellutanum*) was performed mostly by carbohydrate chemists Two polymers were first isolated in the 1930s from *P charlesii* by the Nobel laureate W N Haworth and colleagues (Haworth et al 1937) These polymers were given the trivial names mannocarolose and galactocarolose because they contained mannosyl and galactosyl residues, respectively The carolose root is to honor Charles Thom, the discoverer of the *charlesii* species of *Penicillium* growing on Italian maize Mannocarolose and galactocarolose were among the first small polysaccharides to be used to evaluate the new method of methylation analysis for which W N Haworth later received the Nobel prize in chemistry galactose-containing species. Preston et al. (1969) isolated an extracellular phosphorylated polysaccharide containing mannosyl and galactofuranosyl residues. This polymer was later shown to be a peptidophosphogalactomannan, pPGM. It is now known that *Penicillium fellutanum* produces mainly two classes of complex exocellular galactomannans, i) soluble peptidophosphogalactomannan, (pPGM), and ii) membrane-bound lipo-pPGM (Preston et al., 1969; Rick et al., 1974; Gander et al., 1980). Based on compositional similarity, the membrane-bound lipo-pPGM is likely a precursor of extracellular soluble pPGM (Beachy, 1977; Gander et al., 1980; Gander and Laybourn, 1981).

The structure of the major extracellular pPGM has been examined by wet chemistry and by nuclear magnetic resonance (NMR) spectrometry (Preston et al., 1969; Gander et al., 1974; Rick et al., 1974; Gander, 1977; Unkefer and Gander, 1979; Unkefer et al., 1982; Bonetti and Gander, 1990). A model depicting the major sugars and phosphodiesters of extracellular pPGM structure is shown in Fig. 1.

The basic structure of extracellular pPGM is that of a phosphoglycopeptide (Preston et al., 1969; Gander et al., 1974; Unkefer and Gander, 1979; Unkefer et al., 1982; Bonetti and Gander, 1990). The peptide contains 9 seryl and 7 threonyl residues; it lacks aromatic and sulfur-containing amino acyl residues. All of the threonyl residues have mannooligosaccharides (mannosyl, mannobiosyl, or mannotriosyl), attached through O-glycosidic linkage as do 6 of the 9 seryl units. The mannan backbone is composed of about 20 2-O-α-D-mannotetraosyl units attached by α(1->6)-linkages. Eight to ten galactan chains, each containing up to 30 5-O-β-D-galactofuranosyl residues, are attached to the mannan via α(1->3)-linkages. Approximately 10 phosphocholine residues and the other *N*-methylated derivatives of phosphoethanolamine are attached to C-6 positions of the mannan (Unkefer et al., 1982) The 6-O-phospho-(*N* peptidyl-2 -aminoethanol) residues are attached to C-6 position of galactofuranosyl residues (Bonetti and Gander 1990) by phosphodiester linkages



(M stands for mannopyranosyl G$_f$ stands for galactofuranosyl residues, respectively). The mannan backbone is attached by O-glycosidic linkage to a seryl residue of a peptide which contains about 30 amino acyl units (Rick et al., 1974 Beachy 1977)

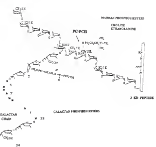
Penicillium fellutanum secretes glycohydrolases catalyzing the depolymerization of complex oligo- and polysaccharides, and other enzymes such as an acid phosphomonoesterase (AP) a nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH), and a bis-(p-nitrophenyl)phosphate phosphodiesterase (Salt and Gander, 1985) A crude enzyme preparation from the culture filtrate of a medium containing 2 mM phosphate catalyzes the removal of phosphocholine from pPGM (Salt and Gander 1985) An exo-β D-galactofuranosidase attacks the galactofuran chains in pPGM from the non-reducing end, releasing galactose monomers, under C-source limited conditions, suggesting that this polymer is a reserve source of carbon nutrient (Rietschel-Berst et al., 1977)

The determination of the structure and physiological function(s) of these peptidophosphogalactomannan species of *P. fellutanum* has been a long term goal in this laboratory. *Penicillium species*, as well as other filamentous fungi, have an ability to adapt to a wide range of temperatures, pH, nutrients and environmental conditions. It has been suggested that these phosphorylated complex polysaccharides may have important roles in fungal physiology and cell growth. This conclusion is based on the previous data that show that the composition of these polymers and the enzymes which modify the polymers are sensitive to nutritional and environmental stimuli (Salt and Gander, 1985). However, how these polymers and their substituents, such as phosphocholine phosphodiester residues, are involved in these processes is unknown. It was observed that this fungus can grow in the medium stressed with up to 3 M NaCl. Thus, in conjunction with the above broad research goal, the specific objectives of this research focused on determining i) the role(s) of phosphocholine residues attached to this polymer in choline metabolism, ii) how this fungus overcomes the high osmotic stress, and iii) whether the phosphocholine in extracellular peptidophosphogalactomannan (pPGM) is involved in this process.

The results of this study will be applicable to our understanding of the roles of other complex cell wall and extracellular polysaccharides in the biochemistry and physiology, especially in stress management, of other organisms.

Figure 1 Model of the major sugars and phosphodiester residues of extracellular peptidophosphogalactomannan (pPGM) structure of *P fellutanum.*

Reducing terminal mannosyl residues are O-glycosidically linked to 3 KDa peptide Complete molecule as isolated, contains 30-32 mannosyl residues, 12 oligomannosides of 1-3 mannosyl units, a mannan of about 80 mannosyl units, 8-10 galactan chains of varying lengths Phosphodiester-linked components are choline and ethanolamine attached to C-6 of mannopyranosyl residues and *N*-peptidyl-phosphoethanolamines attached to C-6 of galactofuranosyl residues For simplicity only one phosphocholine residue is shown in mannan backbone The cleavage site of nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH) is indicated by the arrow


MANNAN PHOSPHODIESTERS
CHOLINE
ETHANOLAMINE
PC PCH
3 KD PEPTIDE
GALACTAN PHOSPHODIESTERS
GALACTAN
CHAIN
PEPTIDE
2
4
7
28
30

CHAPTER 2
REVIEW OF LITERATURE

A common feature of microorganisms and higher organisms is the build-up of a large variety of polysaccharides and glycoconjugates (glycopeptides and glycoproteins). Many of the cell-wall and extracellular polysaccharides and glycopeptides are complex containing hexosyl, pentosyl, uronosyl, and polyol residues, and highly substituted with nonsugar moieties such as acyl, acetal, phosphoryl (monoester or diester), sulfuryl groups. This review will primarily focus on the structural features and the biological and physiological functions of these polymers.

The second half of this review will describe the general aspects of osmoregulation in bacteria and fungi. The importance of choline as a metabolite and its metabolism in fungi will be described in Introduction of Chapter 4.

Structure and Biological/Physiological Functions of Extracellular or Wall Polysaccharides and Glycoproteins

Bacteria

Cell surface carbohydrates of bacteria include extracellular polysaccharides (EPSs), capsular polysaccharides (CPSs), lipopolysaccharides (LPSs), lipooligosaccharides (LOSs), and the periplasmic glucans (membrane-derived oligosaccharides and β-glucans) (Sutherland, 1985; Breedveld and Miller, 1994). By virtue of their location, cell-surface polysaccharides influence the manner in which bacterial cells interact with their environment (Leigh and Coplin, 1992).

Lipopolysaccharides. Lipopolysaccharides (LPSs, endotoxins) of enteric bacteria consist of lipid A, the core oligosaccharide, and the O-specific polysaccharide moiety (O-antigen), with considerable compositional heterogeneities (Rick, 1987, Raetz, 1990). The core oligosaccharide consists of two regions: the inner and outer core. The inner core is generally composed of L-*glycero*-D-*manno*-heptose (heptose), 3-deoxy-D-*manno*-2-octulosonic acid (KDO), phosphate, and ethanolamine residues, and its carbohydrate structure is highly conserved in all enterobacterial LPSs (Holst and Brade, 1992). The outer core contains more common hexoses, such as glucose, galactose, and *N*-acetylglucosamine, and varies slightly among and within bacterial species. The structure of O-antigen is most variable; this polysaccharide is generally composed of repeating units of tetrasaccharides, [abequose-mannose-rhamnose-galactose]$_n$, and may contain various unusual sugars (Rick, 1987, Raetz, 1990). Either the core sugars or the GlcNAc of lipid A are heavily phosphorylated (2 to 5.6 %) as ethanolamine phosphate, ethanolamine pyrophosphate, pyrophosphate, orthophosphate, or ribitol phosphate (Muhlradt et al., 1977, Horton and Riley, 1981, Wilkinson, 1981).

Lipopolysaccharides are endotoxic and stimulatory to the immune systems of animal hosts and are also considered as virulence factors during pathogenesis in animals and plants (Raetz, 1990). However, the function of LPS with respect to the physiology of the bacteria is poorly understood. Lipopolysaccharides bind divalent cations such as Mg^{2+} (Ferris and Beveridge, 1986) and create as effective permeation barrier to hydrophobic substances (Labischinski et al, 1985, Nikaido and Vaara, 1985). They can also act as receptors for phage (Lam et al, 1992).

Capsular Polysaccharides In many bacteria, surface hydrophobicity is augmented by an adherent (often covalently bound) cohesive layer, forming the morphological entity termed the capsule (capsular polysaccharide, CPS Whitfield and Valvano, 1993) Hundreds of serologically different CPSs (K antigens) have been identified in gram-negative bacteria, the serologically nonspecific capsule is called colanic acid or M antigen (formerly called slime, Goebel, 1963) Capsular polysaccharides are usually linear and acidic, and contain acetamido groups, a larger degree of structural heterogeneity is mostly due to the distribution of O-acetyl substituents on these polymers Despite their structural heterogeneity, the fundamental structures of CPSs consist of fairly small, regular repeating sequences of from 1 to 7 saccharide units (Jennings, 1983) *Neisseria meningitidis* has been classified serologically into 8 groups based on the compositional difference of CPSs. Group A CPS is a partially O-acetylated, 1,6-linked homopolymer of 2-acetamido-2-deoxy-D-mannopyranosyl phosphate and groups B and C CPSs are homopolymers of sialic acid (Jennings, 1983) CPSs enhance the virulence of pathogenic bacteria by protecting the cells against phagocytosis and an antibody-dependent cytotoxic killing (Smith, 1977, Feingold, 1969). The CPSs also function as bacteriophage receptors (Lindberg, 1973) promoters of adherence to a variety of surfaces (Costerton et al. 1974), binding and trapping agents of various substances (Rees, 1976), protection against desiccation (Rees, 1976), and inducers of interferon production (Kato et al , 1975)

Periplasmic Glucans in Gram-negative Bacteria The periplasmic space of enteric bacteria contains large quantities (5 to 20% of the total cellular dry weight) of highly anionic polysaccharides, known as either membrane-derived oligosaccharides (MDOs, Kennedy, 1982) or as β-1,2 glucans (Breedveld and Miller, 1994). In *Escherichia coli*, the glucans (MDOs) contain a β-1,2-linked backbone and β-1,6-linked branches, and have 6 to 12 glucose residues. MDOs are linear glucans and highly substituted with nonsugar moieties such as phosphoglycerol, phosphoethanolamine, O-succinyl side chains (Kennedy, 1987). These linear glucans are found in relatively smaller number of bacteria compared with the cyclic β-glucans. The cyclic β-glucans (originally called crown gall polysaccharides) are found almost exclusively in the *Rhizobiaceae* family (Breedveld and Miller, 1994). *Agrobacterium* and *Rhizobium* species synthesize cyclic β-1,2-glucans containing 17 to 40 glucose residues which can be substituted by phosphoglycerol, methylmalonic acid or O-succinic acid (Hisamatsu et al., 1987). *Bradyrhizobium japonicum* synthesizes β-glucans linked by both β-1,3 and β-1,6 bonds, having DPs (degree of polymerizations) ranging from 10 to 13 (Miller et al., 1990), which can be substituted by phosphocholine (Rolin et al., 1992).

These periplasmic glucans, both MDOs and cyclic β-glucans, are known to be involved in osmoregulation of periplasmic spaces of gram-negative bacteria during the growth in hypoosmotic environments (Kennedy, 1982; Miller et al., 1986). The cyclic β-glucans also play important roles during plant colonization (Breedveld and Miller, 1994). Periplasmic glucan-defective mutants of *Rhizobium meliloti* form ineffective white pseudonodules on alfalfa (Geremia et al., 1987), and periplasmic glucan-defective mutants of *Agrobacterium tumefaciens* are avirulent (Puvanesarajah et al., 1985).

Exopolysaccharides (EPSs). Exopolysaccharides are secreted by a variety of bacteria; they can remain associated with the cell wall to form a bound capsule layer, or they can be released into the milieu as extracellular slime (Leigh and Coplin, 1992). The bacterial EPSs can be either homopolymers or heteropolymers and may carry a variety of nonsugar substituents. Although it is known that some low molecular weight poly- or oligosaccharides, including β-1,2-glucans described above, are found in the culture filtrates of many bacteria as EPSs (Sutherland, 1985) this review will be confined to those EPSs which are produced commonly from disparate groups of bacteria and whose biological or physiological functions are known, such as alginate and succinoglycan.

Alginate is an unbranched, linear polysaccharide and a copolymer of β-1,4 linked D-mannuronic acid (M) and its C-5 epimer α-L-guluronic acid (G) (Sutherland, 1985). Alginate is produced by brown sea weeds and certain bacteria (Sutherland, 1985). Bacterial alginates are acetylated on D-mannuronic acid residues, while this is not the case for alginates from seaweeds (Skjåk-Bræk et al., 1986). There is no repeating unit; the molar ratio of the monosaccharides (M/G) and sequence distribution in alginates vary (Haug et al., 1974). Alginates containing blocks of G residues can form strong gels in the presence of Ca^{++}, this property gives alginates significant applicability to industry (Stokke et al., 1991). Alginates produced from *Pseudomonas* species do not contain G blocks while those from seaweed and *Azotobacter vinelandii* contain such structures (Haug et al. 1974).

In *Azotobacter vinelandii*, this polymer plays a crucial role for survival of this organism under adverse environmental conditions such as desiccation (Sutherland, 1985). A mutant impaired in alginate production is unable to form desiccant-resistant cells (Campos et al., 1996). During infection of *Pseudomonas aeruginosa* in human respiratory tract, alginate overproduced from mucoid form of this organism appears to confer increased resistance to phagocytosis (Baltimore and Mitchell, 1982) and a mechanism of adherence that permits the formation of microcolonies in the lung (Lam et al., 1980).

Succinoglycan is an acidic, high-molecular-weight polymer of repeating octasaccharide subunits composed of seven glucose residues, one galactose residue, and carries succinyl, acetyl, and pyruvyl groups (Aman et al., 1981). This polymer is produced by many members of the family *Rhizobiaceae* and other bacteria (Sutherland, 1985). Although the carbohydrate structures are identical, the ester groups differ depending on the producer organisms. A strain of *Zoogloea ramigera* produces an acid EPS containing the same monosaccharides, although in slightly different molar ratios of succinyl hexosyl residues, an succinoglycan (Ikeda et al., 1982). In *Rhizobium meliloti*, this polymer is required for plant invasion (Battisti et al., 1992), the presence of the succinyl group is necessary for the symbiotic role of this polymer (Leigh et al., 1987).

Peptidoglycan. Peptidoglycans (PGs) are composed of disaccharide-peptide repeating units linked together by both glycosidic bonds (β-1,4 linkages between *N*-acetylmuramic acid and *N*-acetylglucosamine residues) and peptide bonds, usually between the carboxyl of a terminal D-alanine residue on one disaccharide-peptide unit and an amino group on another disaccharide-peptide unit (Shockman and Barrett, 1983). Although the chemical composition of the polymer, especially those amino acids within peptide cross-bridges, vary between bacterial species, the basic glycan chains are conserved (Shockman and Barrett, 1983; Severin and Tomasz, 1996).

Peptidoglycan is responsible for maintaining the strength, relative rigidity, shape of the bacterial walls, and for providing protection to the protoplast from external and internal osmotic forces (Shockman and Barrett, 1983). The PG layers serve to sieve out large molecules and to prevent molecules larger than 1.2 kDa from reaching the membrane (Scherrer and Gerhardt, 1971). The reactive groups of PG contribute to the metal ion-binding properties of walls (Beveridge et al., 1982). The PGs are immunogenic (Zeiger et al., 1981).

Teichoic Acids. The walls of most gram-positive bacteria contain one or more secondary polymers, teichoic acids (TAs), which are covalently linked to the main wall structural component, peptidoglycan (PG). Teichoic acids are mainly composed of glycerol phosphate or ribitol phosphate which are substituted to various extent by phosphodiester groups, sugar residues and usually D-alanine ester residues (Ward, 1981). Teichoic acids are classified in two groups: wall teichoic acids which are linked to muramic acid residues of PG as a phosphodiester (Ward, 1981) and lipoteichoic acids which are macroamphiphiles being anchored hydrophobically through the fatty acid residues of their glycolipid component in the outer layer of the cytoplasmic membrane (Fischer, 1990). The hydrophilic part of lipoteichoic acids is usually 1,3-poly(glycerophosphate) with glycosyl (in most cases glucosyl or galactosyl) substituents and O-D-alanyl residues (Fischer, 1990). The teichuronic acid was isolated from the walls of *Bacillus licheniformis* and characterized as a polymer containing equal proportions of *N*-acetylgalactosamine and D-glucuronic acid (Janczura et al., 1961).

The wall teichoic and teichuronic acids make the cell surface acidic and bind cations to provide the correct ionic environment at the cytoplasmic membrane (Heptinstall et al., 1970; Ward, 1981). The wall TAs form part of the receptor for various bacteriophages and can react with specific antibodies and lectins; however, teichuronic acids are not involved in phage binding (Ward, 1981). The teichuronic acids also appear to be non-immunogenic and in this respect resembles the hyaluronic acid capsules of certain streptococci (Hughes et al. 1971). Lipoteichoic acids (LTAs) may be involved in regulating the germination of *Bacillus subtilis* spores; D-alanine ester-free LTA is newly synthesized during the transition state from vegetative growth to spore formation and this alanine-free purely anionic LTA binds strongly to positively charged autolysins and so prevents access of autolysins to the cell wall substrate PGs (Ehrbaum and Sahl, 1987). The wall TAs and LTAs of pneumococci contain choline phosphodiester residues and the presence of choline is critical for the action of amidase (*N*-acetylmuramoyl L-alanine-amidase) (Höltje and Tomasz, 1975). Details on the function of choline in TAs or LTAs are described in Introduction of Chapter 3.

Fungi

The cell wall of fungi including yeasts, in common with gram-positive bacteria and plants, is mainly composed of polysaccharides and glycoproteins and other minor substances such as lipids, melanin, and D-galactosamine polymers (Gander, 1974; Peberdy, 1990). Fungal wall polysaccharides can be divided into two groups based on their presumed function and physical form. The skeletal polysaccharides mesh together to form a net and the spaces in the net are filled by a matrix of other polymers (Hunsley and Burnett, 1968, 1970). The skeletal polysaccharides are water insoluble and highly crystalline homopolymers and include chitin and β-linked glucans. By contrast, the matrix polysaccharides are amorphous, or slightly crystalline, and are mostly water soluble and include α-glucan and glycoproteins (Peberdy, 1990). The fungal cell wall does not have muramic acid-containing peptidoglycan, teichoic acids, or lipopolysaccharides, which are the major polysaccharides of the walls of bacteria. Bartnicki-Garcia (1973) used the information on the major fungal wall polysaccharides for fungal taxonomy. Fungi also secrete a large variety of extracellular polysaccharides; some of them show antigenic properties (Preston et al., 1970; Latge et al., 1994) and some are of great concern for industrial applications (Sandford and Matsuda, 1980). It has been suggested that some of the extracellular polymers represent the degradation products of cell wall polysaccharides (Gander et al., 1980). Their role(s) in fungal physiology other than as reserve sources of carbon nutrient are largely unknown.

Chitin. Chitin is a linear, β-1,4-linked homopolymer of *N*-acetylglucosamine and is a major structural component of fungal cell walls, accounting for up to 60% of the weight of the cell wall (Cabib and Shematek, 1981; Feofilova, 1995). In filamentous fungi, chitin contributes to the structure of both lateral walls and septa. In the yeast, it is mainly found in the primary septum, although a small amount appears to be dispersed throughout the cell wall (Molano et al., 1980). Fungal chitin forms complexes with other cell wall polymers such as uronic acids, chitosan, and β-1,3-, β-1,6-, and β-1,4-glucans (Feofilova et al., 1995). The skeletal components, chitin and β-glucans, generally show no significant turnover during the different phases of growth of a batch culture, despite the presence of chitinase and β-glucanase in the periplasmic space (Santandreu et al., 1984b). The role of chitin on fungal physiology other than as a major component of cell wall is largely unknown. It is well established that the lipo-chitin oligosaccharide (LCO) produced by the members of *Rhizobiaceae* family, also called Nod-metabolite or Nod-factor, is a signal molecule which is essential for nodulation during bacteria-plants symbiosis (Denarie and Cullimore, 1993). Lipo-chitin oligosaccharides consist of a chitin backbone of 3 to 6 β-

1,4-linked *N*-acetylglucosamine residues which are *N* acylated on the nonreducing end (Spaink et al , 1994)

β-Glucans and Mannoproteins The cell wall of yeasts consist of two major components β-glucans and mannoproteins (Ballou, 1976 Fleet 1991 Klis, 1994) The β-glucans of *Saccharomyces cerevisiae* are glucose homopolymers that account for about 50% of the cell wall dry weight (Fleet, 1991) Based on their chemical linkage characteristics, they are subdivided into β-1,3-glucan and β-1 6-glucan (Manners et al , 1973a, 1973b) The β-1 3-glucan consists mainly of linear β-1,3-linked glucan, with β-1 6 linkages at intervals which form branch points The degree of branching is significant in determining the crystallinity and thus the solubility of this polymer This class of glucan has an average of 1 500 glucose residues per molecule and forms insoluble fibers (Manners et al. 1973a) The β-1,6 glucan contains mainly β-1 6-linkages with some β-1,3-linked branch points, and has a DP of about 140 glucose residues (Boone et al , 1990) The β-1 3 glucan and the β-1,6-glucan are cross-linked to each other (Mol and Wessels, 1987) The β-1 6-glucan is associated with chitin (Surarit et al , 1988)

In yeasts, the matrix component of cell wall is composed of mainly two classes of mannan-protein complexes (Ballou, 1976, Fleet, 1991). The first groups are larger molecules, with a mass of greater than 150 kDa, have a branched polysaccharide moiety of up to 150 mannosyl units, the backbone of the molecule is composed of α-1,6-linkages from which short chains with mixed α-1,2- and α-1,3-linkages branch. The mannan backbone is attached to asparagine residues of the protein through *N* glycosidic bonds The phosphorylated, *N*-linked oligosaccharide (called peptidophosphomannan) from *S.*

cerevisiae mannoproteins have been isolated and characterized (Hernandez, 1989) The second groups are smaller molecules some are composed of short α-1,2-/α-1,3-linked mannosides attached to a protein by *O*-glycosidic bonds

Some of the mannoproteins closely interact with the glucan component Some mannoproteins can be extracted from the yeast cell wall by boiling in 20% sodium dodecyl sulfate (SDS) or by 6 M urea whereas others are extracted only after digestion of the cell wall with β-glucanase (Pastor et al , 1984, Valentin et al , 1984 Mrsa et al., 1992) The mannoproteins released by denaturing agents appear to be non-covalently associated with the cell wall The release of some mannoproteins by β-glucanase suggested that these mannoproteins are covalently linked to the wall β-glucan (Pastor et al , 1984) Kapteyn et al (1995) showed, by immunological means, that the β-1,6-glucosylated cell wall mannoproteins of *Candida albicans* contain a β-1,3-glucan epitope, and that β-1,3-glucan and β-1,6-glucan are linked to proteins by phosphodiester linkages It was suggested that the incorporation of proteins into the cell wall is accomplished by extracellular cross-linkage between a β-1,6-glucan-β-1,3-glucan heteropolymer and cell wall proteins. It is known, in *S. cerevisiae*, that several cell surface proteins, such as α-agglutinin, are attached to the plasma membrane through glycosylphosphatidylinositol (GPI) anchors (Wojciechowicz et al , 1993) It appears that α-agglutinin is released from the GPI anchor attached to the plasma membrane and becomes a periplasmic intermediate which subsequently is linked covalently to the cell wall β-1,6-glucan via a transglycosylation reaction (De Nobel and Lipke, 1994 Lu et al 1995). The cell wall β-1,6-glucan is also known to be a receptor for K1 killer toxin secreted from a RNA virus (Hutchins and

Bussey, 1983). Cell wall mannoproteins are involved in pathogenesis, such as immunogenesis and adhesion of *Candida* cells to mammalian tissues (Calderone and Fukayama, 1992; Calderone and Wadsworth, 1993).

The skeletal wall components of most filamentous fungi are the chitin and β-1,3-glucan (Peberdy, 1990). Chitin has not been found in the walls of Oomycetes and Zygomycetes (Aronson, 1965; Hunsley and Burnett, 1970). Oomycetes are the only fungi to have walls containing cellulosic polymer, which is a linear homopolymer of β-1,4-linked glucose residues (Bartnicki-Garcia, 1973). Zygomycetes contain chitosan as a major wall component (Bartnicki-Garcia, 1973). The cell wall of *Neurospora crassa* mainly consists of β-glucan, protein or glycoprotein, and chitin, progressing from the outermost to innermost layer, respectively (Hunsley and Burnett, 1970). The α-1,3-glucan is a straight-chain polysaccharide and is a major matrix component in the walls of most Ascomycetes and Basidiomycetes (Bull, 1970a; Wessels et al., 1972). In some *Aspergillus* species, the α-glucan has alternating α-1,3- and α-1,4-linkages and is known as nigeran. The α-glucan is usually found at the outer surface of the wall and occurs as thick irregular fibrils (Hunsley and Burnett, 1970). While the evidence from other organisms suggests that the α-glucan is a stable wall polymer (Wessels et al., 1972), it serves as reserve source of carbon nutrient in *Aspergillus nidulans* (Zonneveld, 1972a). Glycoproteins are also found in the walls of filamentous fungi; however, they appear to be more heterogenous than those in yeast, both with respect to the polysaccharide component and in the protein-carbohydrate linkage. The polysaccharide component may be a homo- or heteropolymer: the glycoprotein from *Neurospora crassa* is composed of *N*-acetyl glucosamine, galactose and glucuronic acid residues. Both *N*- and *O*-glycosidic linkages have been reported in this polymer (Peberdy 1990)

The cell walls of most *Penicillium* species are composed of glucans (with traces of galactose and mannose) chitin, and proteins (Gander, 1974) Galactofuranose residues are present in the cell wall and extracellular glycopeptides of *P. charlesii* (renamed as *P fellutanum*) (Gander, 1974 Gander et al. 1974) and in the cell wall of *P erythromellis* (Rupérez and Leal, 1981) The alkali-soluble cell-wall polysaccharide of three different strains of *P expansum* was characterized as a α-1,5-D-glucan and the water-soluble polysaccharide was composed of a linear tetrasaccharide repeating unit [-6)-β-D-Gal*f*-(1,5)-β-D-Gal*f*-(1,5)-β-D-Gal*f*-(1 5)-β-D-Gal*f*-(1-]$_n$ (Parra et al., 1994) A galactomannan, with side chains of 1 5-linked β-D-galactofuranosyl residues attached to the mannan, was extracted from hyphae of *Aspergillus niger* (Barreto-Bergter et al , 1980) The β-1,5-galactofuran and α-1 3-glucan were found in the cell-wall material of some other *Penicillium*, *Eupenicillium*, and *Aspergillus* species (Leal et al , 1992) It appears that the presence of β-1,5-galactofuran in the cell wall is characteristic to *Penicillium* and *Aspergillus* species. The galactofuranosyl residues in *Penicillium* cell wall seem to be antigenic determinants of some *Penicillium* species (Preston et al , 1970)

Extracellular Polysaccharides and Glycopeptides of Fungi The extracellular biopolymers from yeast contain remarkable examples of biochemical diversity This review, from yeast extracellular polysaccharides, will be confined to the phosphorylated glycopeptides or polysaccharides.

Phosphomannans are secreted into the culture medium, often in very high yields, by yeasts belonging to the genus *Hansenula* and related genera (Jeanes et al. 1961)

Phosphomannans of yeast include a variety of structural types which have in common the D-mannose 6-phosphate moiety of the phosphodiesters (Slodki, 1962). Strains of *Sporobolomyces* are known that produce O-acetyl-O-phosphogluco-galactans (Slodki 1966). Two types of phosphomannan have been described (Peberdy, 1990). The first type are polysaccharides whose macromolecular structure is primarily dependent on phosphodiester linkages between mannose oligosaccharides, for example, the phosphomannan of *Hansenula capsulata* (P-6-Man-α-1-P-6-Man-α-1-P-6-Man-). The second type includes polysaccharides in which the glycosyl phosphate residues occur exclusively as nonreducing end groups, for example, the polysaccharide of *Hansenula holstii* (P-6-Man-α1,3-Man-α1,3-Man-α1,3-Man-α1,2-Man).

Galactomannans (GMs) are widely distributed among most *Penicillium* and *Aspergillus* species (Azuma et al., 1971; Gander, 1974; Barreto-Bergter and Gorin, 1983). These polymers are constituted mainly of mannose, galactose, and glucose, with minor amounts of protein (Gander, 1974; Barreto-Bergter et al., 1980). They are mainly composed of an α-1,2- and α-1,6-linked D-mannopyranosyl backbone and galactan side chains, some of which are β-1,5-linked D-galactofuranosyl residues (Preston et al., 1969; Gander et al., 1974; Unkefer and Gander, 1982; Barreto-Bergter and Gorin, 1983).

Peptidogalactomannans (PGMs) have been obtained from numerous species of *Aspergillus* (Sakaguchi et al., 1969; Azuma et al., 1971), *Penicillium* (Azuma et al., 1971), and a dermatophyte *Trichophyton mentagrophytes* (Barker et al., 1967). The peptidogalactomannan from dermatophytes differs from those of *Aspergillus* and *Penicillium* in that it contains α-1,4- and lacks α-1,6-linkages (Barker et al., 1967). It is unclear whether these polymers had no phosphoesters attached, or they did not analyze for phosphates.

Peptidophosphogalactomannans (pPGMs) have been found from a yeast *Cladosporium wernecki* (Lloyd, 1970b, 1972) and from *P. fellutanum* (Preston et al., 1969, Gander, 1974, Gander et al., 1980). *Cladosporium wernecki* produces a peptidophosphogalactomannan (pPGM) which is composed of D-mannopyranosyl residues that are mainly 1->2 linked, and smaller amounts of 1->6 and 1->3 substituted mannopyranosyl residues (Lloyd, 1970b). Galactose is found as nonreducing terminal D-galactofuranosyl and galactopyranosyl residues (Lloyd, 1970b). The phosphogalactomannan of this polymer contains phosphodiester groups bridging a side chain containing 7 mannosyl and 1 galactosyl residues to the main polysaccharide core. The main core also contain phosphodiester groups. The peptide of this polymer contains relatively large quantities of seryl and threonyl residues (Lloyd, 1970b), which is similar to the composition of the peptide of pPGM from *P. fellutanum* (Rick et al., 1974).

The pPGM of *P. fellutanum* is highly substituted with nonsugar residues such as phosphocholine and phosphoethanolamine phosphodiester residues (Unkefer and Gander, 1982). Details on the structure of pPGM from *P. fellutanum* is described in Introduction (Chapter 1). A membrane-bound peptidophosphogalactomannan (Lipo-pPGM) has been isolated from *P. fellutanum* and, based on structural similarity, has been considered as a precursor of extracellular pPGM, however, it does not undergo α-, β-elimination in alkali (Benchy, 1977, Gander et al., 1980).

Galactomannans are known to be antigenic and are present in the fluids of patients with invasive aspergillosis (Azuma et al., 1971). Latgé et al. (1994) showed that the α-(1-

2) (1-6)-mannan backbone of galactomannan of *A fumigatus* is not antigenic but galactofuran is immunogenic The pPGM of *P fellutanum* is antigenic (Preston et al 1970) However, the roles of these polymers in fungal physiology are largely unknown.

Osmoregulation

Overview

Cellular adaptation to extreme environmental stress, such as hot, cold, acid, alkaline or saline environments, is a fundamental biological process needed for survival and growth of organisms (Rains et al., 1980; Herbert and Codd, 1986 Ingraham, 1987, Da Costa et al., 1989) Osmotic stress is one such restrictive environmental factor, caused by large concentrations of either salts or non-ionic solutes in the surrounding medium which results in dehydration (Brown, 1978, Yancey et al , 1982, Le Rudulier et al 1984, Blunden and Gordon, 1986 Csonka, 1989 Bloomberg and Adler, 1992, Rhodes and Hanson, 1993, Galinski 1995) Drought and freezing are the other forms of water-related stress causing dehydration (Ingraham, 1987, Potts, 1994) These stresses (salinity, drought, and freezing) are the major limiting factors in agricultural productivity (Le Rudulier et al , 1984) and also of great concern in the food industry Cellular adaptation to osmotic stress is thus an important area for basic research, with possible future applications in agriculture, medicine and industry (Hollaender et al 1979, Rains et al 1980 Blunden and Gordon, 1986)

Microorganisms (Brown, 1978, Csonka, 1989; Galinski, 1995), algae (Blunden and Gordon, 1986), plants (Rhodes and Hanson, 1993) and animals (Zablocki et al ,

1991) have evolved a variety of mechanisms for adapting to these stresses. This review will focus on cellular adaptation to osmotic stress in bacteria and fungi.

Organisms, such as microorganisms, algae, and plants, with a rigid wall structure, maintain an osmotic pressure in the cytoplasm that is higher than that of the surrounding environment, resulting in an outward-directed turgor pressure (Blomberg and Adler, 1992). The proper maintance of turger is essential for cell division and growth (Csonka and Hanson, 1991; Blomberg and Adler, 1992). Since the cell envelope is permeable to water, changes in the environmental osmolarity trigger the flux of water across the cytoplasmic membrane, resulting in lysis under low-osmolarity or dehydration under high-osmolarity growth conditions. Dehydration (i.e. decrease in internal water content) results in a reduction in the turgor pressure and a shrinkage of the cytoplasmic volume. The decrease in the cytoplasmic volume causes the concentrations of the intracellular metabolites to increase, resulting in a reduction in the intracellular water activity (Csonka, 1989). The elevation in the concentrations of various intracellular molecules due to dehydration may be inhibitory to cellular processes; inhibitors of specific enzymes may reach deleterious concentrations (Atkinson, 1969) or the increase in the concentrations of ions may become toxic (Walderhaug et al., 1987). Thus, organisms must possess mechanisms to respond to inhibitory or lethal levels of osmolarity in the environment.

Of various means evolved to overcome osmotic stress, intracellular accumulation of solutes to provide osmotic balance with the environment is a commonly used strategy in a diverse set of organisms (Yancey et al., 1982; Le Rudulier et al., 1984; Wyn Jones, 1984; Grumet and Hanson, 1986; Csonka, 1989; Weretilnyk et al., 1989). Although any solute (called osmolytes) will contribute to the internal water potential, only a restricted group, the so-called compatible solutes, can accumulate to high concentrations without interfering with intracellular enzyme activity and metabolic processes (Brown and Simpson, 1972, Csonka and Hanson, 1991). Compatible solutes do not produce adverse effects upon protein structure and solubility, protein-protein interactions, enzyme-substrate interactions, or protein-nucleic acid interactions, as would high concentrations of inorganic salts (Yancey et al., 1982). Generally, compatible solutes do not carry a net electrical charge near pH 7; this property can be beneficial, because uncharged molecules can accumulate to high intracellular concentrations without greatly disturbing the charge balance and thus the structures of cellular macromolecules (Brown, 1978, Yancey et al., 1982).

These compounds fall into a few classes: sugar alcohols (i.e., polyols: glycerol, sorbitol, etc.) and nonreducing sugars such as sucrose and trehalose, amino acids (proline, glutamate, etc.) and amino acid derivatives (i.e., betaines and other quaternary ammonium compounds, glycine betaine, proline betaine, etc.), peptides, methylamines, and urea (Measures, 1975, Yancey et al., 1982, Blunden and Gordon, 1986, Csonka and Hanson, 1991, Blomberg and Adler, 1992, Galinski, 1995). These compounds may also directly protect or stabilize enzymes and other cellular components from high salt concentrations (Pollard and Wyn Jones, 1979, Yancey et al., 1982, Blomberg and Adler, 1992). Some of these compounds are of great concern in agricultural, industrial, and medicinal applications (Blunden and Gordon, 1986, Potts, 1994).

Intracellular accumulation of compatible solutes (i.e., organic osmolytes) can occur either by *de novo* synthesis or by transport from the environment, depending on each type of solute and the metabolic capability of a given organism. In the case where organic osmolytes are present in the surrounding medium, uptake is usually preferred over synthesis *de novo* (Csonka, 1989; Galinski, 1995). However, even organisms unable to produce their own compatible solutes (e.g., most of the non halophilic bacteria) may acquire a certain range of osmotolerance by the accumulation of external osmolytes. Kennedy (1982, 1987) proposed that the biosynthesis of a class of membrane-derived oligosaccharides (MDO) in *E. coli* is stimulated by the osmotic strength of the environment and that these oligosaccharides may act as polymeric osmoprotectants and play a key role in osmoregulation.

Bacteria

Bacteria frequently encounter osmotic changes in their natural habitat. Osmotic conditions also change significantly during fermentation processes. Because of frequent variations in the availability of water, effective adaptation reactions are required to maintain proper turgor by increasing internal osmolarity, thus preventing cell lysis or dehydration. Adaptation to high osmolarity (osmoregulation) of bacteria is generally accomplished by the intracellular accumulation of organic compatible solutes and K$^+$ ions (Yancey et al., 1982; Epstein, 1986; Csonka and Hanson, 1991). Accumulation of these compounds are dependent on the compositions of culture medium and pH (Csonka, 1989; Ogahara et al., 1995). When such a molecule can be directly shown to protect the organism against inhibitory levels of osmotic stress, it is called an osmoprotectant, the most widespread being glycine betaine (Csonka, 1989).

Gram-negative bacteria. The mechanism of osmoregulation in bacteria has been most intensively studied in *Escherichia coli* and *Salmonella typhimurium* (Cairney et al., 1985; Milner et al., 1988). As a primary response to an osmotic upshift, K$^+$ ions and glutamate accumulate (Booth and Higgins, 1990). In a second step, these substances are replaced by neutral compatible solutes such as glycine betaine, proline, and trehalose (Csonka, 1989; Lucht and Bremer, 1994). Glycine betaine can be taken up from the environment or synthesized *de novo* from its precursor, choline (Landfald and Strom, 1986).

Escherichia coli possesses two transport systems for glycine betaine, ProU, a binding protein dependent system, and ProP, a secondary transport system (Csonka, 1989; Culham et al., 1993). Although enteric bacteria are unable to synthesize glycine betaine, *E. coli* K-12 can convert exogenous choline to glycine betaine with betaine aldehyde as the intermediate by a function of *bet* gene products under conditions of osmotic stress; in this respect, choline is also an osmoprotectant for *E. coli* (Styrvold et al., 1986; Landfald and Strom, 1986; Strom et al., 1986). The individual *bet* gene products include the high affinity transport system, choline dehydrogenase, and betaine aldehyde dehydrogenase (Styrvold et al., 1986; Landfald and Strom, 1986). The *bet* gene products, which are induced under conditions of high osmolarity, can be further induced by the addition of choline to the growth medium, and require aerobic conditions for their expression (Styrvold et al., 1986; Landfald and Strom, 1986). When glycine betaine is absent from the medium, synthesis of trehalose takes place in *E. coli* (Strom and Kaasen, 1993). *Salmonella typhimurium* lacks corresponding genes and therefore is unable to use choline as an osmoprotectant (Andersen et al., 1988). In *E. coli*, choline and glycine betaine can not serve as a carbon or nitrogen source (Le Rudulier and Bouillard, 1983); thus, it appears that the transport of these compounds into enteric bacteria is modulated only by the osmotic strength of the medium (Perroud and Le Rudulier, 1985). In *Rhizobium* species, these compounds can support growth and are also effective osmoprotectants (Bernard et al., 1986). The catabolism of glycine betaine, however, is inhibited in high osmolarity medium (Smith et al., 1988).

In addition to the accumulation of these compatible solutes, the outer membrane porin proteins in *E. coli* are involved in osmoregulation; high osmolarity favors synthesis of the OmpC porin and derepresses OmpF expression (Forst et al., 1987). This regulation is mediated by a two-component signal transduction system consisting of EnvZ, a transmembrane osmosensor, and OmpR, a positive regulator of gene expression (Nonoka et al., 1986; Forst et al., 1987).

Gram-positive bacteria. Gram-positive bacteria usually have a much higher cytoplasmic turgor than gram-negative bacteria and can tolerate higher osmolarities even in the absence of adaptational strategies (Galinski, 1995). Similar to the situation in enterobacteria, *Bacillus subtilus* initially accumulates K^+ ions, which are subsequently replaced by proline and glycine betaine (Kempf and Bremer, 1995); choline exogenously added into the growth medium accumulates as glycine betaine (Boch et al., 1994). *Streptomyces* species accumulate proline, glutamine, and alanine by increased synthesis due to high concentration of salt (Killham and Firestone, 1984). *Listeria monocytogenes*, which is an opportunistic pathogen to human and is of concern to the food industry, use glycine betaine, K^+, proline, and carnitine as major compatible solutes (Ko et al., 1994; Smith, 1996).

Staphylococcus aureus can grow in the environment with up to 3.5 M NaCl and its ability to grow in low-water-activity foods contributes to its important role as an agent of food poisoning (Graham and Wilkinson, 1992). In contrast to enteric bacteria, cytoplasmic pool levels of K do not change much in this organism grown with increasing osmolarity (Graham and Wilkinson, 1992). Choline, glycine betaine, and proline were shown to act as major osmoprotectants for this organism (Graham and Wilkinson, 1992). Choline transport activity of *S. aureus* is repressed by high phosphate concentrations and derepressed by low phosphate concentrations, suggesting that there is a connection between the phosphate and osmotic stress regulons (Kaenjak et al., 1993).

Lactobacillus plantarum preferentially accumulates glycine betaine, when it is present, over other compatible solutes under high osmolarity conditions. During osmotic downshock, glycine betaine, and other osmoprotectants previously accumulated are released rapidly into the growth medium (Glaasker et al., 1996).

In *Lactobacillus plantarum* glycine betaine accumulation was not only observed when the medium osmolarity was high, but significant amounts of glycine betaine also accumulated when the cells were grown without addition of KCl, NaCl, or sucrose (Glaasker et al., 1996). Similar observations have been made for other gram-positive bacteria, but this is not observed for gram-negative species. This difference may be related to the fact that, in general, gram-positive bacteria maintain a higher turgor pressure than gram-negative strains (Csonka, 1989). Compatible solutes that do not interfere with essential cellular functions are therefore crucial in regulation of high turgor pressure, particularly in gram-positive bacteria (Glaasker et al., 1996).

Fungi

Fungi, in common with other walled organisms, require a positive cell turgor pressure, in excess of a critical value, to permit cell expansion and thus growth. Thus, the osmotic pressure of the environment is a fundamental determinant of fungal growth

(Luard, 1982 Blomberg and Adler 1992) Many fungi are capable of growth in media of increased osmotic pressure resulting from high concentrations of salts or sugars (Brown, 1976) in which any increase in external osmolarity must be offset by a comparable change in intracellular osmolarity (Brown and Simpson, 1972 Yancey et al , 1982) In most fungi including yeasts, glycerol and other polyhydroxy alcohols, such as erythritol (Gadd et al 1984) and arabitol (Adler and Gustafsson, 1980 Nobre and Da Costa, 1985) are the major organic compatible solutes (Brown, 1978, Blomberg and Adler, 1992). The increased production of polyols under increasing external osmolarity appears to be a response common to many halotolerant and xerotolerant fungi (Jennings, 1983)

Many polyols, such as arabitol in osmotolerant yeasts and mannitol in many filamentous fungi, are present in high concentrations regardless of the external water potential and could be regarded as constitutive compatible solutes (Harris, 1981) These higher polyols, which are less permeable across the membrane than glycerol and thus are easier to retain, may serve to protect cellular structures against desiccation stress (Blomberg and Adler 1992) and may serve as carbohydrate reserves and as a storage form of reducing power (Lewis and Smith, 1967)

Yeasts Osmoregulation studies in fungi were performed mainly with yeasts. The natural habitats of the xerotolerant yeasts include floral nectaries, the yeasts are commonly associated with bees and honey If honey is fermented, these yeasts are usually responsible Their commercial significance lies primarily in the food industry since they can cause spoilage of wine must, syrups and conserves, fruit juices, dessert wines, dried fruits, molasses and malt extract They are also used in the preparation of various oriental fermented foods including soy sauce and miso paste. Their habitats therefore normally place them in high osmolarity environments (Brown, 1978; Barnett et al., 1983).

Production of polyols is common among fungi; analysis of the pattern of polyol production can be used for fungal taxonomy (Pfyffer et al., 1990). Oomycetes do not produce polyols; proline serves as the main compatible solute in these fungi (Pfyffer et al., 1990). The intracellular polyol content of *Zygosaccharomyces rouxii* and *Saccharomyces cerevisiae* increased with decreasing external water potential by addition of polyethylene glycol 200, glucose (Brown, 1974) or NaCl (Brown, 1978). The internal concentrations under these stresses reached molal levels. *Debaryomyces hansenii*, which grows in medium containing up to 4.1 M NaCl (Blomberg and Adler, 1992), accumulates glycerol, as well as K$^+$, in parallel with the external concentration of NaCl (Gustafsson and Norkrans, 1976; Gustafsson, 1979). Return of cells to more dilute media resulted in rapid release of glycerol in amounts that were proportional to the decrease of external solute concentration (André et al., 1988). This work also provided more direct evidence for a role for glycerol in osmoregulation. Externally supplied glycerol reestablished ability to grow under high osmolarity condition for a mutant of *Debaryomyces hansenii* with a lowered capacity to produce glycerol (André et al., 1988). In *Hansenula anomala*, glycerol, arabinitol and trehalose were detected as the main internal organic solutes. Glycerol and trehalose prevailed in *Saccharomyces cerevisiae*; of these solutes only glycerol responded to growth-medium salinity (Ballinger and Larher, 1988). Although many organisms use amino acids and their derivatives as compatible solutes (Yancey et al., 1982), there has been no report concerning the significance of these solutes as osmoresponsive compounds in yeasts (Brown and Stanley, 1972 Adler and Gustafsson, 1980)

Filamentous fungi. Osmoregulation in filamentous fungi has relatively rarely been studied The major internal polyols of filamentous fungi tested are glycerol, erythritol, arabitol, and mannitol (Hocking and Norton, 1983) Glycerol is the major osmoresponsive compatible solute within the mycelia grown under low water potential (generated by various stress solutes) in filamentous fungi including *Penicillium ochrochloron* (Gadd et al. 1984) and *Aspergillus nidulans* (Beever and Laracy, 1986) Although glycerol is the predominant compatible solute in growing cells of yeasts and filamentous fungi, it is not the solely osmoresponsive polyol in filamentous fungi (Jennings 1984 Blomberg and Adler 1992) The type of polyol that accumulates depends on culture age, nutrient conditions type of stress solute used and the organism studied (Jennings, 1984 Blomberg and Adler, 1992) Accumulation of erythritol, with glycerol as the major polyol, in *Penicillium* and *Aspergillus* species examined, progressively increased with increasing external solute concentration (Adler et al , 1982, Gadd et al., 1984, Beever and Laracy 1986) and decreased after osmotic down-shock (Beever and Laracy 1986). In *Penicillium* and *Aspergillus* species, there has been no report concerning the significance of amino acids and their derivatives, such as betaines and choline derivatives, as osmoregulatory compatible solutes

CHAPTER 3
RECYCLING OF PHOSPHOCHOLINE FROM EXTRACELLULAR PEPTIDOPHOSPHOGALACTOMANNAN INTO CYTOPLASMIC CHOLINE DERIVATIVES IN PENICILLIUM FELLUTANUM

Introduction

Fungi produce complex polysaccharides and glycopeptides which are membrane-bound, cell wall-bound or extracellular (Jeanes et al., 1961, Slodki, 1962, Gander, 1974, Ballou, 1976, Berkeley et al., 1979, Sandford and Matsuda, 1980). Although the structural chemistry of fungal polysaccharides (Gander, 1974, Barreto-Bergter and Gorin, 1983) and their industrial applications (Berkeley et al., 1979, Sandford and Matsuda, 1980) were well reviewed, their role(s) in fungal physiology are mostly unknown. Some extracellular polysaccharides or glycopeptides of fungi may have a role as reserve sources of carbon (Bull, 1972, Kohama et al., 1974, Tuekam and Gander, 1993) and are known to be involved in animal and plant pathogenesis (Anderson, 1978, Dow and Callow, 1979, Halverson and Stacey, 1986). Some of these polymers are biologically active and known to be antigenic (Preston et al., 1970, Notermans and Soentoro, 1986, Latgé et al., 1994).

Ubiquitous filamentous fungi belonging to the genera of *Penicillium* or *Aspergillus* produce soluble extracellular polysaccharides and glycopeptides, called peptidophosphogalactomannans (pPGMs) (Gander, 1974, Leal et al., 1992). *Penicillium fellutanum* (formerly *P. charlesii* G. Smith) produces mainly two classes of complex exocellular polysaccharides, i) soluble peptidophosphogalactomannan (pPGM) with a mass of about

70 kDa, and ii) membrane-bound lipo pPGM (Preston et al , 1969; Rick et al , 1974 Beachy 1977 Gander et al , 1980, Gander and Laybourn, 1981) Based on the compositional similarity, lipo-pPGM is likely a precursor of extracellular soluble pPGM and may be another variation of anchor glycoprotein in that pPGMs have structural features similar to the glycosylphosphatidylinositol (GPI) anchor molecules (Low, 1989 Thomas et al , 1990) The structure of the major extracellular pPGM has been examined by wet chemistry and by NMR spectroscopy (Rick et al 1974 Gander et al., 1974, Unkefer and Gander 1979 Unkefer et al. 1982, Bonetti et al. 1993) The mannan backbone contains phosphoethanolamine residues and about 10 α-D-mannopyranosyl 6-phosphocholine phosphodiester residues, galactofuran chains contain galactofuranosyl 6-O-phospho-(*N*-peptidyl-2 aminoethanol) phosphodiester residues (Unkefer et al , 1982) A model depicting the major sugars and phosphodiesters of extracellular pPGM structure is shown in Fig 1 in Chapter 1

Some microbial cell wall or extracellular polymers are known to contain phosphocholine phosphodiesters. Examples are teichoic and lipoteichoic acids of several viridans group of streptococci and clostridia (Klipper Balz et al 1985, Podvin et al , 1988 Tomasz, 1967), pPGM of *Penicillium fellutanum* (Unkefer et al. 1982) and the cyclic β-(1 6)-β-(1 3)-glucans of *Bradyrhizobium japonicum* (Rolin et al , 1992) Although phosphocholine phosphodiester is a structural component of these biological polymers its physiological role is largely unknown. Interest in the role of these components of cell surface or extracellular polysaccharides on biosynthesis and depolymerization of cell wall components by biosynthetic and autolytic enzymes has increased following the work of Tomasz and his colleagues. They showed that phosphocholine in cell wall lipoteichoic acid is necessary for autolysin amidase to catalyze the cleavage of peptidoglycan muramoyl residues in *Streptococcus pneumoniae* (Mosser and Tomasz, 1970; Holtje and Tomasz, 1975). Mutants incapable of converting teichoic acid phosphoethanolamine diesters to phosphocholine diesters show abnormal morphology because choline is required in activation of the amidase that catalyzes hydrolysis of peptidoglycan; ethanolamine does not substitute for choline. *Steptococcus oralis* grown on a choline-free medium had abnormal morphology; the doubling time was about 2.5-fold longer than cells grown with added choline (Horne and Tomasz, 1993). About 80-90% of the choline taken up from the medium was incorporated into cell wall teichoic acid and 10% was located in plasma membrane.

The physiological role(s) of the phosphodiester residues such as phosphocholine in extracellular pPGM of *P. fellutanum* are unknown. The specific objective of the study reported in this chapter was to determine the role(s) of phosphocholine released from the extracellular pPGM under low phosphate conditions on the physiology of *P. fellutanum*. The results demonstrate that phosphocholine in extracellular pPGM is an extracellular precursor of intracellular choline-O-sulfate and glycine betaine.

Materials and Methods

Chemicals and Strain

L-[*methyl*-^{13}C]methionine, [2-^{13}C]glycine, and deuterium oxide (D_2O) were purchased from Sigma Chemical Co. and Aldrich Chemical Co., USA. Sodium (trimethylsilane)-1-propanesulfonate (TSP) was obtained from Wilmad Glass Co N J, USA. Choline chloride, glycine betaine (GB), and phosphocholine (Na) were from Sigma Choline-O-sulfate (COS) was synthesized according to the method of Stevens and Vohra (1955) and its purity was confirmed by ^{1}H-NMR spectrometry All other chemicals were reagent grade obtained from commercial sources *Penicillium fellutanum* (previously *P charlesii* G Smith NRRL 1887) was originally from Dr Kenneth Raper Department of Bacteriology University of Wisconsin, Madison, Wisconsin, and maintained for over 15 years in the laboratory

Media and Growth Conditions

The conidiospores for liquid shake culture were harvested from cultures grown on Czapek-Dox agar medium for 12 days at room temperature under constant white light. Conidiospore suspensions were prepared in sterile water solution containing 0 9% NaCl (w/v) and 0 125% Tween 20 (w/v) as described by Beachy (1977) For the production of pPGM, *P fellutanum* was grown in either the standard growth (SG) medium containing 20 mM phosphate or low phosphate standard growth (LPSG) medium containing 2 mM phosphate Media compositions are presented in Appendix A Cultures were grown at 22°C under constant light using 500 ml notched wide mouth Erlenmeyer flasks containing ~10^{6} conidiospores in 200 ml of medium with constant shaking at 40 rpm on a New Brunswick Model G-10 shaker

Enzyme Assays

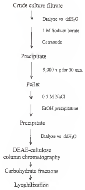
Two enzyme activities, nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH) and acid phosphomonoesterase (AP) were determined daily during the growth of cultures Enzymes were assayed with a 20 min incubation at 22°C, in a reaction mixture of 500 µl of 10 mM sodium citrate buffer (pH 3 0 for AP or pH 5 0 for PC PCH), 2 0 µmoles of *p*-nitrophenyl phosphocholine for PC PCH or *p*-nitrophenyl phosphate for AP, and 100 µl of culture filtrate with ddH_2O to total volume of 850 µl The reactions were stopped by addition of 1 ml of 0 2 N NaOH *p*-Nitro-phenol released was determined at 410 nm in a Hewlett Packard Model A Diode Array Spectrophotometer

Isolation and Fractionation of Extracellular Soluble pPGMs

Soluble pPGMs were obtained from 2 to 4 liters of culture filtrates of 8-day old cultures in SG or LPSG medium Procedures for the isolation of extracellular soluble pPGMs followed the methods established by Gander et al (1974) (Fig. 2) The cultures were filtered *in vacuo* through Whatman No 4 filter paper dialyzed overnight against tap water and then against ddH_2O using MWCO 14 000 dialysis tubings. The retentate was treated with 5% by volume of 1 M borate (adjusted to pH 7 0 by adding 1 N NaOH) and then 4% by volume of 5% (w/v) cetyltrimethylammoniumbromide. The gummy pellets obtained after centrifugation at 9,000 x g for 30 min at 4°C using BECKMAN Model J2-21 Centrifuge were dissolved in 0 5 M NaCl solution, precipitated and washed with 3 volumes of absolute ethanol, and dialyzed overnight against ddH_2O

Figure 2. Flow diagram of procedures for isolation of extracellular peptidophosphogalactomannan (pPGM).

See text or Gander et al. (1974) for details


Crude culture filtrate
Dialyze vs ddH₂O
1 M Sodium borate
Cetramide
Precipitate
9,000 x g for 30 min.
Pellet
0.5 M NaCl
EtOH precipitation
Precipitate
Dialyze vs ddH₂O
DEAE-cellulose column chromatography
Carbohydrate fractions
Lyophilization

The crude pPGM preparations were applied to DEAE-cellulose column preequilibrated with 50 mM $K_2B_4O_7$ and eluted in stepwise manner with 1 L of each 50 mM $K_2B_4O_7$, ddH$_2$O, 0.01 N HCl/0.06 M LiCl, 0.01 N HCl/0.4 M LiCl, and 0.5 M NaCl. Fractions of 100 μl were assayed for total carbohydrate following Dubois phenol-H_2SO_4 method with 0.242 mM glucose solution as a reference by measuring absorbance at 490 nm (Dubois et al., 1956). The carbohydrate-containing fractions were pooled, dialyzed against ddH$_2$O, frozen at -20°C, and then freeze-dried *in vacuo*. The schematic flow diagram for isolation and purification of pPGM is presented in Fig. 2. Unless otherwise stated, the extracellular pPGMs used in these experiments are from the solutes eluted with 0.01 N HCl/0.06 M LiCl from a DEAE-cellulose (DE-52) column. This fraction of pPGM contains more phosphocholine diesters than occurs in other fractions of pPGM (Salt and Gander, 1985).

Preparation of [*methyl*-^{13}C]pPGM by Metabolic Labeling

Penicillium fellutanum was cultured for 8 days in 4 liters of SG medium enriched with L-[*methyl*-^{13}C]methionine (50 mg/200 ml) and ^{13}C-methyl-labeled pPGM was isolated from the culture filtrate. The general procedures for the isolation of pPGMs are described above. Thirty hours after inoculation, 50 mg of L-[*methyl*-^{13}C]methionine dissolved in ddH$_2$O was filter sterilized (0.22 μm, Millipore) and added to each culture flask. Since extracellular pPGM obtained from high phosphate medium (SG) contains more phosphocholine residues than those obtained from low phosphate medium (LPSG) (Salt and Gander, 1985), [*methyl*-^{13}C]pPGM was obtained from the culture filtrate of SG medium. The [*methyl*-^{13}C]pPGM obtained was analyzed by proton-decoupled ^{13}C-NMR spectroscopy.

Release of Phosphocholine from Extracellular pPGM

The ^{13}C-methyl-labeled pPGM obtained was used to follow the release of phosphocholine from extracellular pPGM. Two hundred milligrams of purified [*methyl*-^{13}C]pPGM were dissolved in 4 ml ddH$_2$O, and added 72 h after inoculation, by filtration through 0.22 µm membrane filter (Millipore), to 200 ml cultures in LPSG medium. This time was selected to include the 3-8 day interval of maximum PC PCH activity. At day 8, the cultures were harvested and the [*methyl*-^{13}C]pPGM added was recovered from the culture filtrates through a modified isolation procedure (see below) and analyzed for the compositional changes by proton-decoupled ^{13}C-NMR spectroscopy. The same quantity of unlabeled pPGM was used as control.

Ten milliliters of 1 M sodium borate (pH 7.0) were added to 200-ml culture filtrate from each culture flask. The negatively charged [*methyl*-^{13}C]pPGM bound to DE-52 matrix was preequilibrated with 50 mM $K_2B_4O_7$. The column was washed with the same solution to remove residual glucose in the culture filtrate; [*methyl*-^{13}C]pPGM was then eluted with 0.01 N HCl/0.4 M LiCl. The carbohydrate-containing fractions were dialyzed against ddH$_2$O, freeze-dried *in vacuo*, and dissolved in 4 ml ddH$_2$O and analyzed for any compositional changes by ^{13}C-NMR spectroscopy. The intracellular solute pools of mycelia obtained from the same cultures were examined for the existence of any intracellular ^{13}C methyl labeled substance(s).

Preparation of Mycelial Extracts

Intracellular solute pools of mycelia were analyzed by proton decoupled ^{13}C-NMR spectroscopy after extraction with 80% ethanol. Culture conditions are described above. Fifty milligrams of either L-[*methyl*-^{13}C]methionine or [2-^{13}C]glycine were added to each 200-ml culture at 30 h which is the approximate time of germination. [2-^{13}C]Glycine was used to biologically label carbons in choline and its derivatives. It was noted that PC PCH activity gradually decreased after day 6. Therefore, according to the activity profile of PC PCH in culture filtrates collected daily, unless otherwise stated, all the mycelial extracts for subsequent experiments were obtained from the cultures grown for 8 days. Mycelia were obtained by vacuum filtration through C-type sintered glass filters. Mycelia were washed with water and then were treated with 80% ethanol for 24 h at room temperature and centrifuged at 8000 x *g* for 30 min at 4°C. Insoluble debris were removed by vacuum filtration through M-type sintered glass filters. The filtrates were evaporated *in vacuo* to near dryness at 40°C with enough time to completely remove any residual ethanol. The dried materials were dissolved in 4 ml ddH$_2$O and water-soluble substances were analyzed by ^{13}C-NMR spectroscopy.

^{31}P-NMR Spectroscopy

To examine the compositional changes in pPGMs isolated from the cultures, proton-decoupled ^{31}P-NMR spectrometry was performed at 121 MHz on a NT 300 spectrometer with Fourier transform mode without spinning. One hundred fifty milligrams of each sample were dissolved in 4ml ddH$_2$O with 0.5 ml D$_2$O added. The chemical shift, (0.22 ppm), of phosphocholine diester attached to C-6 position of mannopyranosides in pPGM was used as internal reference: this chemical shift was established by Unkefer et al (1982). Acquisition parameters are described in the legends of each Figure.

^{13}C-NMR Spectroscopy of Mycelial Extracts and pPGMs

Natural-abundance proton-decoupled ^{13}C-NMR spectra were recorded at 75.45 MHz using a NT-300 spectrometer with a 7 Tesla Oxford magnet operating in the pulsed Fourier transform mode. Unless otherwise stated, all samples were examined at 22°C with broad-band decoupling of protons, with full nuclear Overhauser enhancement (NOE), and without sample spinning. Four milliliters of each mycelial extract were analyzed by ^{13}C-NMR spectroscopy with addition of 0.5 ml D$_2$O containing 2% TSP as internal reference. Frequencies and acquisition parameters are described in the legends of each Figure.

Synthesis of Choline-O-sulfate

Choline-O-sulfate (COS) was synthesized according to the methods of Stevens and Vohra (1955). Four grams of choline chloride (Sigma) were dissolved in 10 ml concentrated H$_2$SO$_4$ and heated in a tightly sealed vial at 100°C for 5 h. After cooling the mixture at room temperature overnight, 80 ml of absolute ethanol were added and the alcoholic mixture was stored at 4°C overnight. The resulting white crystals were obtained by filtering the mixture through F-type sintered glass filter and by washing with additional

200 ml of absolute ethanol to remove any remaining sulfate or choline. The white crystals obtained were dissolved in 15 ml of 80% ethanol and recrystallized after freeze drying *in vacuo*. One hundred seventy one milligrams of dried, white crystals were obtained. The purity and identity of synthesized COS were confirmed by ^{13}C-NMR spectroscopy.

Identification of COS and Glycine Betaine by ^{13}C-NMR Spectroscopy

Initially, authentic COS, glycine betaine, choline chloride, and phosphocholine (20 mM each) were dissolved in ddH_2O and their chemical shifts were compared to those of signals in mycelial extracts. However, because some chemical shifts depend on environment, it was necessary to dissolve the authentic chemicals in mycelial extracts, instead of ddH_2O, to create the same environment as the molecules present in the extracts. Thus, to the mycelial extract obtained from the 8 day grown mycelia in LPSG medium and transferred to a 12 mm NMR tube, authentic solid glycine betaine, COS, and choline chloride (30 mM each) were added sequentially. ^{13}C-NMR spectra were obtained after each addition and were analyzed for the influence of the added authentic substance on the signal intensities. In some experiments, [2-^{13}C]glycine instead of L-[*methyl*-^{13}C]methionine was added to the culture (50 mg/200 ml of medium) to metabolically label a significant percentage of all carbons in choline of extracellular pPGM and those carbons of the two choline derivatives found in intracellular solute pools.

Results

Production of Phosphoesterases in Low Phosphate and High Phosphate Medium

Low levels of nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH) and an acid phosphomonoesterase (AP) were detected in high phosphate medium (SG, 20 mM NaH_2PO_4) (Fig. 3A). However, in low phosphate medium (LPSG 2 mM NaH_2PO_4) much higher levels of AP and PC PCH activities per ml of culture filtrate were observed (Fig. 3B). This suggests that the high level of PC PCH activity in low phosphate culture medium may be involved in recruiting phosphorus by releasing phosphocholine from extracellular pPGM. The activity of PC PCH persisted for a longer period of time than that of AP in LPSG medium.

^{31}P-NMR Spectra of pPGMs Isolated from High Phosphate or Low Phosphate Medium

The proton-decoupled ^{31}P-NMR spectrum presented in Fig. 4A represents that of pPGM obtaiand from high phosphate (SG) medium and Fig. 4B represents that of pPGM obtained from low phosphate (LPSG) medium. The chemical shifts of each signal on ^{31}P NMR spectra of pPGM have been established by Unkefer et. al. (1982) and Bonetti et al (1993). In Fig. 4A, from right to left, the chemical shifts for phosphocholine attached to mannose, phosphomonomethyl ethanolamine, phosphoethanolamine attached to mannose, *N* peptidyl-phosphoethanolamine phosphodiesters attached to galactan chains are 0.22, 0.82, 0.96, 1.13, 1.30, 1.45 ppm, respectively.

Figure 3 **Production of phosphoesterases by *P. fellutanum* in A) high phosphate medium and B) low phosphate medium.**

Two to three milliliters of culture filtrates were passed through Whatman No. 4 paper and immediately assayed for a nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH) and an acid phosphomonoesterase (AP) Symbols ○, AP; □, PC PCH

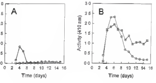
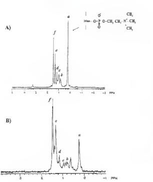

A
B
Activity (410 nm)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
0
2
4
6
8
10
12
14
16
Time (days)

Figure 4. Proton-decoupled ^{31}P-NMR spectroscopy of the pPGMs

The pPGMs were isolated from either A) high phosphate (SG) medium or B) low phosphate LPSG medium. One hundred fifty milligrams of each pPGM were dissolved separately in 4 ml ddH$_2$O with 0.5 ml of D$_2$O. Each signal represents *a* (at 0.22 ppm) phosphocholine attached to mannose, *b* (at 0.82 ppm), phosphomonomethyl ethanolamine attached to mannose, *c* (at 0.96 ppm) phosphoethanolamine attached to mannose, *d*, *e*, *f* (at 1.13, 1.30 and 1.45 ppm, respectively) *N*-peptidyl-phosphoethanolamines attached to galactan chains of pPGM. The chemical shift (0.22 ppm) of phosphocholine in pPGM (indicated by arrow) was used as chemical shift reference for other signals, as established by Unkefer et al. (1982).


A)
f
e
d
c
b
a
Man–O–P–O–CH2 CH2–N+–CH3
O-
O
CH3
CH3
5
4
3
2
1
0
−1
−2
−3
PPM
B)
f
e
d
c
b
a
3
2
1
0
−1
PPM

The ratios of the signal intensities between phosphocholine at 0.22 ppm and a signal at 1.13 ppm, which was arbitrarily selected for comparison of signal intensities, significantly decreased in pPGM isolated from low phosphate LPSG medium (Fig. 4B). Salt and Gander (1988) demonstrated that a crude preparation of PC PCH catalyzed the release of phosphocholine from pPGM. These results, in conjunction with the high level production of PC PCH and AP in low phosphate conditions (Fig. 3), suggest that phosphocholine in extracellular pPGM may be a reserve source of phosphate under phosphate-deficient conditions.

Preparation of [*methyl*-^{13}C]pPGM by Metabolic Labeling

The ^{13}C-NMR spectrum of Fig. 5A represents normal pPGM isolated from SG medium and that of Fig. 5B represents the ^{13}C-methyl labeled pPGM ([*methyl*-^{13}C]pPGM) isolated from SG medium supplemented with L-[*methyl*-^{13}C]methionine. The chemical shifts of each signal in the ^{13}C-NMR spectra of pPGM have been established by Unkefer et al. (1982). In Fig. 5A, the signal at 56.83 ppm (designated as *a*) represents methyl carbons of phosphocholine attached to C-6 of mannopyranoside residues. The methyl carbon signal at 47.24 ppm (designated as *b*) was tentatively identified as that of *N,N'*-dimethyl-phosphoethanolamine (Unkefer and Gander, 1979) attached to C-6 of other mannopyranoside residues in pPGM. In Fig. 5B, the signal intensities of methyl groups of phosphocholine and *N,N'* dimethyl phosphoethanolamine significantly increased, indicating that the methyl carbons of both choline and dimethyl ethanolamine of extracellular pPGM were metabolically enriched with ^{13}C by adding L-[*methyl*-^{13}C]methionine into the growing cultures. The purified [*methyl*-^{13}C]pPGM was then used as a potential source of ^{13}C-methyl-enriched metabolites in the soluble cytoplasmic fraction (see below).

Release of Phosphocholine from Extracellular pPGM

To more clearly demonstrate that phosphocholine in extracellular pPGM is cleaved during the growth of *P. fellutanum* in low phosphate medium and to trace the released phosphocholine, the purified [*methyl*-^{13}C]pPGM shown in Fig. 5B was added to the low phosphate (LPSG) cultures and recovered from the culture filtrates 5 days later and then analyzed by ^{13}C-NMR for any compositional changes (Fig. 5C). The two signals, representing ^{13}C-labeled phosphocholine (56.83 ppm) and *N,N*-dimethyl-phosphoethanolamine (47.24 ppm), completely disappeared from the added and reisolated [*methyl*-^{13}C]pPGM. It was also shown that the signals at ~ 110 and 84 ppm, which represent galactofuranosyl residues (Unkefer et al., 1982), disappeared from the polymer (Fig. 5C). Instead, significant signals at 95 and 99 ppm that are not in intact pPGM appeared.

Cytosolic Localization of Methyl Carbons of Phosphocholine from Extracellular pPGM

Intracellular solute pools of mycelia obtained from cultures initially containing 2 mM phosphate and either unsupplemented (Fig. 6A), supplemented with [*methyl*-^{13}C]pPGM (Fig. 6B) or supplemented with unlabeled pPGM (Fig. 6C) were surveyed for the presence of any ^{13}C labeled substance(s). Each extract contained the same concentration of TSP (0.22%) as a reference for integration of peak intensities of individual signals. The spectral window of 0 to 60 ppm covers the chemical shift range of methyl carbons. Signals that resonated at 56.77 and 56.23 ppm (designated as *P* and *Q* respectively) in each spectrum, are at positions in the region expected for -N^{+}-trimethylated carbons. The signal at 54.99 was not identified. The signal at 48.76 ppm is likely that of a dimethyl substance (Unkefer et al., 1982). Signals in the region of 20 to 45 ppm may be those of monomethyl substances.

With the TSP signal as reference, comparison of relative intensities of signal *P* and *Q* of Fig. 6B with those in Fig. 6A and 6C showed that the relative intensities of signals *P* and *Q* were about 4.3-fold and 2.3-fold larger than those in the extract from the culture supplemented with unlabeled pPGM (Fig. 6C). These data show that *P* (56.77 ppm) and *Q* (56.23 ppm) signals in Fig. 6B are the ^{13}C-enriched carbons and that they originated from the extracellular ^{13}C-methyl-labeled phosphocholine of the [*methyl*-^{13}C]pPGM added to the culture.

This result suggests that at least the methyl groups of phosphocholine from extracellular pPGM were relocalized as a component of intracellular methyl carbon containing solutes. Addition of unlabeled pPGM to the culture (Fig. 6C) resulted in a small increase in signals *P* and *Q*, compared with those in Fig. 6A.

Identification of Intracellular Choline-O-sulfate and Glycine Betaine

Identification of the two intracellular methyl-containing substances was performed by proton-decoupled ^{13}C-NMR spectroscopy.

Figure 5. Proton-decoupled ^{13}C-NMR spectra of extracellular pPGMs.

pPGMs were isolated from the filtrates of A) day 8 culture in high phosphate (SG) medium or of B) day-8 culture in the same medium but supplemented with L-[*methyl*-^{13}C]methionine (50 mg/200 ml of medium) 30 h after inoculation. C) The [*methyl*-^{13}C]pPGM was added to low phosphate (LPSG) medium (200 mg/200 ml of medium) on day 3, re-isolated from the culture filtrate 5 days later, and then subjected to ^{13}C-NMR spectroscopy. Spectra were recorded with 13,476, 18,557, and 13,649 acquisitions for A), B), and C), respectively, and with 90° radio frequency pulses of 26 µs applied at 4 s intervals. The signals at 56.75 (designated as *a*) and 47.68 ppm (designated as *b*) represent the methyl carbons of phosphocholine and *N,N*-dimethyl-phosphoethanolamine, respectively, attached to C-6 of mannopyranoside residues in pPGM. Signals were previously assigned by Unkefer et al. (1982).

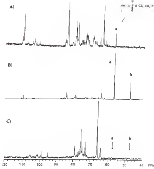
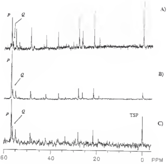

A)
Man O P O CH₂ CH₂ N CH₃
CH₃
CH₃
a
B)
a
b
C)
a
b
120
110
100
90
80
70
60
50
40
PPM

Figure 6. Proton-decoupled ^{13}C-NMR spectra of mycelial extracts.

Mycelial extracts were obtained from 200 ml culture grown in A) low phosphate LPSG medium and in the same medium supplemented with either B) 200 mg of [*methyl*-^{13}C]pPGM or C) 200 mg of unlabeled pPGM. All cultures were harvested 8 days after inoculation and the extract shown in B) spectrum was surveyed for the presence of any ^{13}C-enriched substances in the intracellular solute pools. Either ^{13}C labeled or unlabeled pPGM was added 72 h after inoculation. To 4 ml of each extract 0.5 ml D_2O containing 2% TSP was added. Sodium (trimethylsilane)-1-propanesulfonate (TSP) was used as internal reference for the chemical shifts and also as reference for integrating the peak intensity of each signal. Integration was performed with Felix for Windows 1.02 software (Molecular Simulations Inc.) and relative values were calculated by setting the intensity of TSP signal to 1.0. The integrated values were 8.64, 1.42, and 1.00 for *P*, *Q*, and TSP, respectively, in B) spectrum, those in C) spectrum were 2.00, 0.63, and 1.00 for *P*, *Q*, and TSP, respectively. Spectra were recorded with 1,000 acquisitions, 90° radio frequency pulses of 35 µs applied at 4 s intervals for all three spectra. Symbols *P*, 56.77 ppm, *Q*, 56.23 ppm.


P
Q
A)
P
Q
B)
P
Q
TSP
C)
60
40
20
0
PPM

The chemical shifts of methyl carbon signals of authentic choline chloride and glycine betaine (Fig. 7A) dissolved in ddH_2O were close to the value of 56.23 ppm observed in ^{13}C-methyl-labeled mycelial extracts (Fig. 7C and 7D), that of authentic choline-O-sulfate (COS) dissolved in ddH_2O was close to 56.77 ppm (Fig. 7B) (also see Table 1). Supplementing the growing cultures with L-[*methyl*-^{13}C]methionine resulted in enrichment of methyl carbons of intracellular methyl-containing solutes (Fig. 7D), as did ^{13}C-methyl-labeled pPGM ([*methyl*-^{13}C]pPGM) as shown in Fig. 6B. Although supplementing the culture with L-[*methyl*-^{13}C]methionine was good enough to monitor the fluctuation of methyl-containing substances in intracellular solute pools, it did not serve to monitor the other carbons (e.g., -CH_2-, -COOH, etc.) of the same methyl-containing substances. This problem was resolved by supplementing the culture with [2-^{13}C]glycine which labeled all other carbons, as well as the methyl carbons, of choline and its derivatives. Mycelial extracts from these cultures had two ^{13}C-labeled singlets of N^+-methyl carbon signals at 56.77 and 56.23 ppm, and four ^{13}C-labeled triplets. The triplet signals occurred at 64.90, 67.68, 69.04 ppm, and 172.03 ppm (not shown on the spectrum) regions, suggesting that these signals are related to the methyl carbon signals at 56.77 and 56.23 ppm.

The signals with chemical shifts of 56.77, 64.90, and 67.68 ppm (designated as *P*, *P*1, and *P*2, respectively) were almost equivalent to 56.76 [-N^+-$(CH_3)_3$], 64.78 (-CH_2O-SO_3), and 67.60 ppm (-CH_2-N^+-) of authentic COS (Fig. 7B), indicating that the signal at 56.77 ppm is for -N^+-methyl groups of COS. The chemical shifts of the two unknown *Q* and *Q*2 signals (at 56.23 and 172.04 ppm, respectively) appeared to be equivalent to those of authentic glycine betaine dissolved in ddH$_2$O (Fig. 7A) which has chemical shifts, 56.43 [-N$^+$-(CH$_3$)$_3$], 67.78 (-CH$_2$-N$^+$-), and 172.00 ppm (-COOH). However, the chemical shift of the unknown *Q*1 signal (at 69.04 ppm) in mycelial extract (Fig. 7E) showed about 1.2 ppm difference from the signal for -CH$_2$-N$^+$- (at 67.78 ppm) of authentic betaine dissolved in ddH$_2$O (Fig. 7A). This inconsistency was resolved by dissolving the authentic reference chemicals directly into a mycelial extract instead of ddH$_2$O, thereby creating the same environment for the added authentic chemicals as those of biological intracellular unknown substances (see below).

Dissolving authentic crystalline glycine betaine (to 30 mM) directly into a mycelial extract (shown in Fig. 8A) resulted in a significant increase in three signals at 56.23, 69.04, and 172.04 ppm (designated as *G*1, *G*2, and *G*3, respectively) (Fig. 8B). These results demonstrated that the three signals (*Q*, *Q*1, and *Q*2) in Fig. 7 and Fig. 8A are equivalent to the three signals (*G*1, *G*2, and *G*3, which are the signals of authentic glycine betaine) in Fig. 8B, indicating that the unknown three signals (*Q*, *Q*1, and *Q*2) in mycelial extracts are the signals of biological glycine betaine. Thus these results, taken together, showed that the mycelia of *P. fellutanum* contain intracellular glycine betaine derived from extracellular pPGM. It also showed that the chemical shift of the methylene carbon of authentic glycine betaine is quite different when dissolved in ddH$_2$O (Fig. 7A) compared with that obtained when dissolved in mycelial extracts (Fig. 8B). Subsequently, to the mycelial extract containing added authentic glycine betaine (Fig. 8B), solid authentic COS (30 mM) was dissolved. This resulted in an increase of the three signals at 56.77, 64.90, and 67.68 ppm (designated as *COS*1, *COS*2, and *COS*3 of authentic COS), indicating that the three unknown signals are those of biological COS in the mycelia (Fig. 8C). Finally authentic choline chloride (30 mM) was dissolved in the mycelial extract containing authentic glycine betaine and COS. This resulted in, as shown in Fig. 8D, further increase of 56.77 ppm signal and appearance of two new signals resonating at 58.59 and 70.22 ppm, which were not observed in mycelial extract (Fig. 8A). This indicates that there was no detectable biological choline in the mycelia. These results suggest that phosphocholine (and possibly *N,N*-dimethyl-phosphoethanolamine) which disappeared from extracellular pPGM (see Fig. 5B and 5C), are converted primarily to COS and glycine betaine inside the mycelia grown for 8 days in low phosphate medium.

Discussion

A Role for Extracellular pPGM in Storage and Recycling of Choline and Phosphate

Penicillium fellutanum produces at least two classes of pPGM, extracellular pPGM and cytoplasmic membrane-bound lipo-pPGM (Gander, 1974; Gander and Laybourn, 1981). Although it was suggested (Tuekam and Gander, 1993) that extracellular $pP_{10}GM$ ("10" stands for 10 phosphodiester residues) had a role in survival of *Penicillium* when some nutrients became depleted, the selective removal of a component of pPGM and uptake of that component from the medium have not been demonstrated previously. It was observed that, under low phosphate condition, phosphocholine disappeared from extracellular pPGM (Fig. 5). The methyl groups of COS and glycine betaine, labeled with carbon 13 derived from the [*methyl*-^{13}C]pPGM, accumulated as soluble constituents in mycelia (Fig. 6).

Figure 7 Proton-decoupled ^{13}C-NMR spectroscopy of mycelial extracts for identification of intracellular choline derivatives.

The chemical shifts of authentic chemicals, A) betaine chloride (30 mM) and B) COS dissolved in ddH$_2$O (100 mM), were compared to those of signals in mycelial extracts. The mycelial extracts were obtained from 200 ml each of the LPSG medium enriched either with C) 50 mg of [2-^{13}C]glycine or with D) 50 mg of L-[*methyl*-^{13}C]methionine. Cultures were grown for 8 days and ^{13}C-labeled materials were added on day 3. To increase the concentration of intracellular solutes, the mycelial extract in E) spectrum was obtained from 1.6 liters of the same medium with no addition of any labeled materials. After freeze drying, all mycelial extracts were dissolved in 4 ml ddH$_2$O with 0.5 ml of D$_2$O containing 2% TSP as internal reference of chemical shift. Spectra were recorded at 75.45 MHz with A) 868, B) 1,636, C) 500, D) 1,000, and E) 2,916 acquisitions, pulse width 35 μs (90°), acquisition time 204.80 ms at 22°C. Symbols: *P*, 56.77; *Q*, 56.23 ppm.

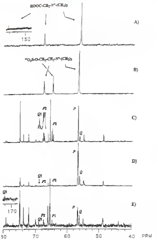

HOOC-CH$_2$-N$^+$-(CH$_3$)$_3$
150
A)
$^-$O$_3$S-O-CH$_2$-CH$_2$-N$^+$-(CH$_3$)$_3$
B)
Q1
P2
P2
P
Q
C)
P
Q
Q1
P2
P2
D)
Q2
170
P2
Q1
P1
P
Q
E)
80
70
60
50
40
PPM

Figure 8. Identification of intracellular choline-O-sulfate and glycine betaine.

Spectra A through D (proceeding from the bottom to the top)represent A) mycelial extract, B) mycelial extract plus added authentic solid glycine betaine chloride (GB). C) mycelial extract plus added GB and choline-O-sulfate (COS), D) mycelial extract plus added GB, COS and choline chloride. The final concentrations of each authentic chemical was 30 mM. Spectra were recorded after 2,000 acquisitions, pulse width 35 μs (90°), acquisition time 204.80 ms Symbols: *P*, 56.77 ppm, *Q*, 56.23 ppm, *G1*, $-N^{+}-(CH_3)_3$, *G2*, $-CH_2-N^{+}-$, *G3*, -COOH of glycine betaine, respectively: *COS1*, $-N^{+}-(CH_3)_3$, *COS2*, $-CH_2O-SO_3$, *COS3*, $-CH_2-N^{+}-$ of choline-O-sulfate, respectively: *Cho1*, $-N^{+}-(CH_3)_3$, *Cho2*, $-CH_2OH$, *Cho3*, $-CH_2-N^{+}-$ of choline chloride, respectively

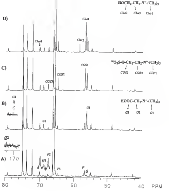

HOCH2-CH2-N+-(CH3)3
Cho2
Cho3
Cho1
D)
Cho1
Cho3
Cho2
C)
-O3S-O-CH2-CH2-N+-(CH3)3
COS3
COS2
COS1
COS1
COS2
COS3
B)
G3
172.38
HOOC-CH2-N+-(CH3)3
G3
G2
G1
G1
G2
Q2
A) 170
P2
Q1
P1
P
Q
80
70
60
50
40
PPM

Table 1 Comparison of the chemical shifts of authentic chemicals dissolved in mycelial extract or ddH$_2$O

	GB			COS			Chol		
	-CH$_3$	-N-CH$_2$	-COOH	-CH$_3$	-N-CH$_2$-	-CH$_2$OSO$_3$	-CH$_3$	-N-CH$_2$-	-CH$_2$OH
extract[a]	56.23	69.04	172.04	56.77	67.68	64.90	–	–	–
GB[b]	56.25	69.00	172.02						
COS[b]				56.79	67.61	64.85			
Chol[b]							56.77	70.22	58.59
GB[c]	56.43	67.78	172.00						
COS[c]				56.76	67.60	64.78			
Chol[c]							56.38	69.85	58.18

[a] Mycelial extract obtained from the culture grown for 8 days in low phosphate LPSG medium (see Fig. 7E and 8A)). [b] Authentic chemicals dissolved in mycelial extract. [c] Authentic chemicals dissolved in ddH$_2$O. Symbols GB, Glycine betaine, COS, Choline-O-sulfate, Chol, Choline; -, No signals, -N-, -N$^+$-.

Choline did not accumulate significantly as a soluble intermediate in the mycelia (Fig. 8) Choline-O-sulfate (COS) is known as a storage form of choline and sulfate in filamentous fungi (Markham et al., 1993) The results suggest that extracellular peptidophosphogalactomannan (pPGM) is a reserve source of choline in *P. fellutanum* However, these data do not exclude other role(s) for phosphocholine and phosphoethanolamine diester residues of pPGM in fungal physiology No significant enrichment of methyl carbons of either COS or glycine betaine occurred in a similar experiment performed in a medium containing 20 mM phosphate instead of 2 mM phosphate (data not shown) These data, coupled with the several-fold increase in acid phosphomonoesterase and nonspecific phosphocholine diester phosphocholine hydrolase activities in cultures initially containing a) 2 mM phosphate (Fig. 3B), compared with those of b) 20 mM phosphate (Fig. 3A) are consistent with the following *Penicillium fellutanum* has a mechanism for recycling phosphate and for conserving excess choline not required for synthesis of new membranes Choline-O-sulfate is a primary storage form of choline; however, glycine betaine may be a secondary storage form To my knowledge, this is the first time that an extracellular choline-containing complex polysaccharide has been demonstrated as a precursor of intracellular COS, glycine betaine, or other choline derivatives

The overall process of recycling specific nutrients from complex polysaccharides is likely associated with secretion of a nonspecific phosphocholine diester phosphocholine hydrolase, and an acid phosphomonoesterase to accommodate phosphate depletion from the culture medium. Coupling the release of choline with that of phosphate from a storage polymer, pPGM, is consistent with synthesis of membranes which are major sinks for both phosphate and choline derivatives. Possibly small quantities of other important metabolites are also covalently attached to pPGM and can be released when culture medium becomes depleted of this component. This type of recycling of nutrients back into the organism provides a means of conserving important nutrients or metabolites until they are needed and also a means of selectively releasing them. This may be a general mechanism for providing for continued survival and growth in a nutritionally unbalanced environment.

^{13}C-NMR Analysis of Intracellular Solute Pools by ^{13}C Metabolic Labeling

Since L-[*methyl*-^{13}C]methionine labeled only the methyl carbons and the signals of natural abundant carbons of intracellular choline derivatives were so small, it was difficult to recognize the existence of natural abundant ^{13}C signals. This problem was overcome by adding [2-^{13}C]glycine instead of L-[*methyl*-^{13}C]methionine. It caused the appearance of triplets whose resonances result from a combined singlet and doublet, indicating the presence of three labeled species: a doublet resulted from ^{13}C-^{13}C splitting of signals in those of adjacent ^{13}C-labeled carbons such as -$^{13}CH_2$- and -$^{13}CH_2O$- or -^{13}COOH of choline derivatives (Fig. 7C). A singlet, in the middle of the doublet, would occur in those molecules with adjacent ^{12}C-^{13}C residues (Pasternack et al., 1994). The triplet signals as well as two singlets in Fig. 7C were enough to suggest that the mycelial extracts of *P. fellutanum* contained choline derivatives. The triplet signal at 172.00 ppm in Fig. 7C, which represents -COOH of glycine betaine was too small to be plotted. The results of this study, thus, showed that α carbon of [2-^{13}C]glycine is incorporated into all carbon species of choline and its derivatives (COS and glycine betaine) indicating that exogenous glycine can be used as the sole source of one-carbon pools for the synthesis of choline and its derivatives in *P. fellutanum*. In similar studies, Pasternack et al. (1994) reported that ^{13}C-labeled choline ([1,2,3-^{13}C]choline) was metabolically synthesized from [2-^{13}C]glycine which was the sole exogenous source of one-carbon units in *S. cerevisiae*. this process involves [2,3-^{13}C]serine synthesis from [2-^{13}C]glycine. The serine would result in labeling of both carbons of ethanolamine.

CHAPTER 4

GLYCINE BETAINE AS AN ALTERNATE RESERVE FORM OF CHOLINE UNDER LOW PHOSPHATE AND LOW SULFATE CONDITIONS IN PENICILLIUM FELLUTANUM

Introduction

Choline occurs in nature as an intermediary metabolite mainly for the synthesis of phosphatidyl choline in most eukaryotic organisms. The importance of choline as the precursor of phosphatidyl choline rests in its structural role as the component of plasma, and other, membranes and in its physiological role in the regulation of membrane fluidity (Ellar, 1978, Jonston et al., 1982). Choline is also known as the structural component of some microbial cell wall or extracellular polymers as phosphodiester residues, including teichoic and lipoteichoic acids of streptococci and clostridia (Mosser and Tomasz, 1970, Podvin et al., 1988, Horo and Tomasz, 1993), extracellular peptidophosphogalactomannan (pPGM) of *Penicillium fellutanum* (Unkefer et al., 1982), and the membrane-bound cyclic β-(1,6)-β-(1,3)-glucans of *Bradyrhizobium japonicum* (Rolin et al., 1992). Furthermore, choline is involved in osmoregulation in that it is the precursor of an osmoprotectant, glycine betaine, in many bacteria and plants (Csonka, 1989, Rhodes and Hanson, 1993). In filamentous fungi, choline is the precursor of a sulfate storage molecule, choline-O-sulfate (COS), in sulfate metabolism (Harada and Spencer, 1960, Hussey et al., 1965, Gravel, 1976). Choline influences mycelial morphology by stimulating hyphal extension and by inhibiting branch initiation (Trinci, 1984, Wiebe et al., 1989). Choline is also an essential nutrient for the growth of some filamentous fungi such as those of choline requiring mutants of *Neurospora crassa* (Horowitz et al., 1945) and *Aspergillus nidulans* (Markham and Bainbridge, 1979).

Like most higher eukaryotic organisms and yeasts, for the synthesis of phosphatidyl choline filamentous fungi have both Bremer-Greenberg methylation pathway using precursors provided by endogenous central metabolism and Kennedy cytidine nucleotide pathway using exogenous choline and its derivatives (Horowitz et al., 1945, Kennedy and Weiss, 1956, Bremer and Greenberg, 1961, Steiner and Lester, 1970, Markham and Bainbridge, 1979). However, despite its great functional diversity and importance in cell physiology and metabolism, the sources of the cytosolic pool of free choline in filamentous fungi are not fully understood. Choline-O-sulfate is known to be an endogenous reserve source of choline by stimulating further growth in choline-requiring mutants of *A. nidulans* inhibited because of insufficient choline (Markham and Bainbridge, 1992). However, under choline-deficient conditions, the growth of *A. nidulans* double mutants, carrying both the *cho*A1 mutation and any one of the sulfur-metabolism defective mutations *s*-1, *s*-3, *s*-12, or *s*-50, was not reduced compared to that of the *cho*A1 single mutant (Arst, 1968). This observation led Markham et al. (Markham et al., 1993) to suggest that mutants blocked in sulfate metabolism cannot synthesize COS, such residual growth is due to choline supplied from some other form of endogenous storage molecule. This chapter focuses on an alternative intracellular reserve form of choline in *P. fellutanum*.

Penicillium fellutanum (formerly *P. charlesii* G. Smith) produces mainly two classes of complex exocellular glycopeptides, i) soluble peptidophosphogalactomannan, (pPGM), and ii) membrane-bound lipo-pPGM (Preston et al., 1969, Gander et al., 1980; Rick et al., 1974). The soluble exocellular pPGM contains about 10 α-D-mannopyranosyl 6-phosphocholine residues within the mannan backbone (Unkefer et al., 1982). In Chapter 3, it was demonstrated that phosphocholine of extracellular pPGM is a precursor of intracellular choline-O-sulfate and glycine betaine. Interestingly, it was also shown that no detectable accumulation of free choline in mycelia was observed.

The study in this chapter was thus aimed at determining whether this fungus has other form(s) of intracellular choline, which are utilized for the synthesis of phosphatidylcholine. The result of this study would be helpful in solving the problems shown in those choline-requiring mutants as described above and also for understanding of general aspects of choline metabolism in fungi. For this purpose, I attempted to detect free choline by exposing the mycelia, which contained significant level of COS, to either sulfate-limiting or sulfate sufficient conditions in the low phosphate medium. This approach was based on the fact that COS is a sulfate and choline storage molecule in filamentous fungi (Markham et al. 1993). It was expected that, under sulfate-limiting and low phosphate condition, at least a measurable level of accumulation of choline would be observed. The results suggest that glycine betaine is another intracellular reserve form of choline in *P. fellutanum*.

Materials and Methods

Strains and Growth Conditions

L-[*methyl*-^{13}C]methionine and D_2O were purchased from Sigma Chemical Co USA. Sodium (trimethylsilane)-1 propanesulfonate (TSP) was obtained from Wilmad Glass Co , N J , USA All other chemicals were reagent grade obtained from commercial sources *Penicillium fellutanum* (previously *P charlesii* G Smith NRRL 1887) was from Dr Kenneth Raper, Department of Bacteriology, University of Wisconsin, Madison, Wisconsin and maintained for over 15 years in this laboratory Media compositions are described in Appendix A. For SO_4-limiting conditions, Na_2SO_4 was omitted and $FeSO_4$ 7 H_2O was replaced by $FeCl_2$ 4 H_2O, resulting in SO_4-limited culture medium containing only trace amount of sulfate as micronutrients $Cr_2(SO_4)_3$ 12 H_2O, 0 93 mg/liter and $CuSO_4$ 5 H_2O, 1.6 mg/liter High-sulfate culture medium represents the same low phosphate medium (LPSO, 2 mM Pi) plus 5 mM Na_2SO_4. General culture conditions are the same as described in Chapter 3

Preparation of Mycelial Extracts

Since the extracts obtained by 3 M perchloric acid treatment contained significant signal of carbonate which interfered with the data acquisition during ^{13}C-NMR spectroscopy (not shown), extractions were performed with either absolute or 80 % ethanol, depending on amount and hydration of harvested mycelia. Procedures for ethanol extraction are described in Chapter 3 After extraction and drying *in vacuo*, the residue was dissolved in 4 ml of deionized water and the water-insoluble materials bound to the wall of flask were discarded. The water soluble mycelial extracts were analyzed by proton-decoupled ^{13}C-NMR spectroscopy.

Effect of Phosphate Concentration on the Levels of COS and GB

Cultures were grown in either high phosphate medium (SG) containing 20 mM phosphate or low phosphate medium (LPSG) containing 2 mM phosphate. The mycelia were harvested on day-8 and their intracellular soluble solute pools were analyzed by ^{13}C-NMR spectroscopy after ethanol extraction. The amount of intracellular COS and GB was determined by integrating individual ^{13}C-NMR signal of the spectrum, TSP was used as the reference. Integration was performed with Felix for Windows 1.02 software (Molecular Simulations Inc.).

Mycelial Dry Weight

The mycelial dry weight (D.W.) was determined by taking the mean value of triplicate samples. Mycelia obtained from each 200 ml culture grown for 8 days were filtered *in vacuo* through M-type sintered glass filter with the preweighed, demoisturized Whatman No. 4 paper filter on it. The mycelia on the paper filter were then separately placed in each beaker and heated for 24 h at 110°C.

Effect of Sulfate Concentration on the Levels of COS and GB

To more clearly monitor the fluctuation of intracellular choline derivatives due to different concentration of sulfate in the culture medium, cellular methyl carbons were labeled with ^{13}C by pre-incubating the mycelia for 5 days in LPSG medium supplemented with L-[*methyl*-^{13}C]methionine (50 mg/200 ml medium). These ^{13}C-methyl labeled mycelia were harvested by filtering through sterile M-type sintered glass filter *in vacuo* and washed with 200 ml of fresh LPSG medium modified to contain limiting sulfate. The washed mycelia were then transferred aseptically to 200 ml of the same sulfate-limiting LPSG medium. The transferred mycelia were vigorously suspended and incubated for an additional 8 days. Subsequently, mycelia previously grown in sulfate-limiting LPSG medium were transferred to LPSG medium modified with the addition of 5 mM Na_2SO_4 and cultured for 5 days. The mycelial extracts were analyzed by ^{13}C-NMR spectroscopy.

^{13}C-NMR Spectroscopy of Mycelial Extracts

Mycelial extracts were analyzed by proton-decoupled ^{13}C-NMR spectroscopy as described in Chapter 3. Four milliliters of each mycelial extract was analyzed with addition of 0.5 ml D_2O containing TSP as internal reference. Frequencies and acquisition parameters are described in the legends of each Figure.

Results and Discussion

Levels of Intracellular COS and GB Increase under Low Phosphate Conditions

The mycelial extracts were obtained from the cultures grown for 8 days in three different batches of 200 ml of either low phosphate medium (LPSG) containing 2 mM phosphate or high phosphate medium (SG) containing 20 mM phosphate. With known concentration of TSP (0.22 %) and by integrating each ^{13}C-NMR signal of the spectrum, it was shown that the mycelia grown for 8 days in low phosphate medium contain glycine betaine (GB) (1.5 mg/ g D.W. of mycelia) and about 1.5-fold more COS (9.3 mg/ g D.W. of mycelia) than the mycelia grown in high phosphate medium (Table 2). Unlike those mycelia grown in low phosphate medium, the mycelia grown for 8 days in high phosphate medium accumulated no significant level of GB. It was thus proposed that, under lower phosphate condition, less intracellular choline is required for synthesis of components such as phospholipids in membrane, resulting in accumulation of free choline. However, it was shown in Chapter 3 (see Fig. 8D and Table 1) that no significant quantity of choline accumulated in these mycelia. This suggests that choline accumulates as its derivatives, COS and/or GB.

Time Dependent Accumulation of Choline-O-sulfate and Glycine Betaine

Mycelia grown for 5 days in low phosphate medium enriched with L-[*methyl*-^{13}C]methionine, accumulated ^{13}C-methyl-labeled COS at 56.77 ppm and two unknown signals at 54.94 and 48.75 ppm regions (Fig. 9B). It was confirmed that these three signals are the ^{13}C-methyl-labeled carbon signals by comparing with the signals of mycelial extracts obtained from the same cultures supplemented with unlabeled L-methionine (data not shown). Unlike those mycelia grown for 5 days, ^{13}C-methyl-labeled GB resonating at 56.23 ppm was observed in 8 day-grown mycelia (Fig. 9A).

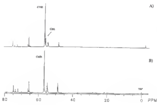
Table 2 Effect of the concentration of phosphate on the accumulation of intracellular choline derivatives

Medium[a] ([Pi], mM)	Growth[b] (g dry weight/ 200 ml medium)	Choline derivatives (mg/g dry weight)[c]	
		COS	GB
LPSG (2)	3.46 ± 0.35	9.3 (0.93)[d]	1.5 (0.15)
SG (20)	4.36 ± 0.21	6.4 (0.64)	ND

[a] Cultures were grown for 8 days and the water-soluble materials of mycelial extracts were analyzed by ^{13}C NMR spectroscopy. [b] Means of triplicates ± SD, in g/200 ml culture medium. [c] The amounts of COS and GB were determined by integrating individual signal on ^{13}C NMR spectra, using Felix for Windows 1.02 software (Molecular Simulations Inc.), where TSP was used as reference. [d] Numbers in brackets represent % COS and GB of mycelial dry weight. Symbols: LPSG: low phosphate medium; SG, high phosphate medium (Medium compositions are described in Appendix A, COS: choline-O-sulfate; GB: glycine betaine; ND, no detectable signal

Figure 9 Time-dependent accumulation of intracellular choline derivatives.

Penicillium fellutanum was grown for A) 8 days, or B) 5 days in low phosphate medium supplemented with L-[*methyl*-^{13}C]methionine. The water-soluble mycelial extracts were analyzed by proton-decoupled ^{13}C-NMR spectroscopy with TSP as internal reference for chemical shifts. *GB*1 represents the signal of ^{13}C-enriched-methyl carbon of glycine betaine. *COS*1 represents the signal of ^{13}C-enriched methyl carbon of choline-O-sulfate (COS). The signal assignment for both glycine betaine and COS was described in Chapter 3


A)
B)
TSP
80
60
40
20
0
PPM

The signals at 56.77 and 56.23 ppm represent those of methyl carbon of COS (designated as *COS*1) and GB (designated as *GB*1), respectively. The signals for COS, GB, and choline were assigned previously (Fig. 8 and Table 1 in Chapter 3). The accumulation of COS preceded that of GB. This suggests that the accumulation of GB may depend upon the depletion of certain nutrient(s) in the culture medium.

No accumulation of choline was observed in either set of mycelia. Since methyl carbon of choline resonates at the same chemical shift (56.77 ppm) as that of COS, the presence or absence of choline was determined by observing two signals resonating at 58.59 ppm and 70.22 ppm regions in mycelial extracts, which represent those of -CH$_2$OH and -CH$_2$ N$^+$-, respectively, of choline.

Effect of Sulfate-deficiency on Accumulation of Choline-O-sulfate and Glycine Betaine.

Since COS is a sulfate and choline storage molecule in filamentous fungi (Markham et al., 1993), it was expected that significant level of choline would accumulate under sulfate limiting and low phosphate condition. To more clearly monitor the fluctuation of the signals, the mycelia were initially ^{13}C-labeled from L-[*methyl*-^{13}C]methionine and used for subsequent experiments. The resulting ^{13}C-labeled, 5-day old mycelia (Fig. 10A) were harvested aseptically from separate culture flasks and transferred to fresh, low phosphate medium as a control (Fig. 10B) and to the same fresh medium but containing only a trace amount of sulfate (Fig. 10C). The transferred ^{13}C-labeled mycelia were cultured an additional 8 days and then their mycelial extracts were subjected to the ^{13}C-NMR analysis with 0.51% of TSP as a chemical shift reference. As shown in Fig. 10B, the mycelia transferred to fresh LPSG medium (as a control) showed appearance of GB (*GB*1 at 56.23 ppm represents methyl carbon of GB) and slightly decreased level of COS (designated as *COS*1, *COS*2, and *COS*3) compared with Fig. 10A. However, the mycelia transferred to and grown in sulfate-limiting fresh medium (Fig. 10C) showed that, while the level of COS significantly decreased, GB level significantly increased to the level as high as that of COS in the mycelia before transfer. Again, no detectable accumulation of choline was observed in these mycelial extracts. These results clearly indicated that COS is a sulfate-storage molecule in *P. fellutanum* as well as in other filamentous fungi, and that GB originates from COS under sulfate-limiting and low phosphate condition.

Effect of High Sulfate on Accumulation of Choline-O-sulfate and Glycine Betaine

To determine whether the levels of GB and COS accumulated are influenced by higher concentration of sulfate in the culture medium, the mycelia previously grown in sulfate-limiting LPSG medium as shown in Fig. 10C, which contained low level of COS and very high level of GB, were transferred to a fresh LPSG medium plus 5 mM SO_4^{2} (i.e., high-sulfate medium) or to a same fresh sulfate-limiting LPSG medium as control.

The signals designated as *COS*1 and *GB*1 in Fig. 11 represent ^{13}C-labeled methyl carbons of COS and GB, respectively. The methyl carbon signal of COS (*COS*1) significantly increased with concomitant decrease of that of GB (*GB*1) in the mycelia grown under high sulfate and low phosphate medium (Fig. 11B), whereas the high ratio of *GB*1 to *COS*1 remained relatively unchanged in control mycelia (Fig. 11A).

Figure 10. Influence of low concentration of sulfate on the accumulation of intracellular choline-O-sulfate and glycine betaine.

Mycelial extracts were analyzed by proton-decoupled ^{13}C NMR spectroscopy. A) the intracellular solute pools of mycelia grown for 5 days in 200 ml low phosphate medium (LPSG) enriched with 50 mg of L-[*methyl*-^{13}C]methionine. These 5 day-grown, ^{13}C-methyl labeled mycelia were transferred aseptically to B) 200 ml of the fresh LPSG medium as control, or to C) the same but sulfate limiting medium. The transferred mycelia were cultured for an additional 8 days. Spectra were recorded at 75.45 MHz with 1,000 scans, pulse width 35 µs (90°), acquisition time 204.80 ms. The final concentration of TSP was 0.51%. The signals designated as *GB*1, *GB*2, and *GB*3 (inset) represent -N^+-$(CH_3)_3$, -CH_2-N^+-, and COOH of glycine betaine, respectively. The signals designated as *COS*1, *COS*2, and *COS*3 represent -N^+-$(CH_3)_3$, -CH_2-O-SO_3-, and -CH_2-N^+- of choline-O-sulfate (COS), respectively.

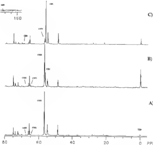
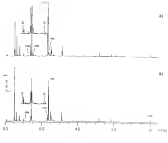

160
C)
B)
A)
80
60
40
20
0
PPM

Figure 11. Influence of high concentration of sulfate on the accumulation of choline-O-sulfate and glycine betaine.

The mycelia previously grown in sulfate-limiting, low phosphate (LPSG) medium (see Fig. 10C) were transferred to A) fresh high sulfate medium (LPSG plus 5 mM Na_2SO_4), or to B) the same sulfate-limiting, LPSG medium as control. The mycelia were harvested 5 days after transfer and their extracts were analyzed by ^{13}C-NMR spectroscopy with the same conditions as described in Fig. 10. The region of 72.50 to 56.80 ppm was replotted by 2-fold enlargement (insets). "X" at 58.59 and 70.22 ppm indicates the absence of the signals representing the carbons of CH_2OH and $-CH_2-N^+-$ of choline, respectively. For signal assignment, see Table 1 in chapter 3.


A)
X
X
B)
X
X
80
60
40
20
0
PPM

Still, no significant accumulation of choline was observed, when compared with the levels of COS and GB in either set of mycelia. These results suggest that COS and GB are metabolically closely related and that they are interconvertible probably *via* choline as the intermediate metabolite, depending upon the concentrations of sulfate and phosphate in the culture medium. Considered together, these results suggested that choline accumulates as COS and GB in the mycelia. The data also clearly indicate that, under sulfate-limiting and low phosphate condition, GB accumulates, no choline was detected. Under high sulfate and low phosphate condition, COS accumulates; no choline was detected in *P. fellutanum*.

Is Glycine Betaine an Intracellular Alternative Reserve of Choline?

Choline-O-sulfate hydrolyzed by choline sulfatase should primarily leave choline as a direct product. Since we used low phosphate, LPSG, medium, the relatively expensive Kennedy cytidine nucleotide pathway making phosphatidylcholine from choline might have been slowed down, causing accumulation of choline. However, under sulfate-limiting condition, GB accumulation significantly increased with concomitant disappearance of COS (Fig. 10C) and *vice versa* under higher sulfate condition (Fig. 11A), suggesting that GB accumulation may originate from COS and *vice versa*. It is speculated that sulfate-limiting conditions may at least partially inhibit the COS biosynthesis steps such as choline sulfotransferase reaction using 3'-phosphoadenosine-5'-phosphosulfate (PAPS) and choline as substrates (Kaji and Gregory, 1959, Harada and Spencer, 1960, Spencer and Harada, 1960, Spencer et al., 1968), causing further accumulation of choline. However, the results of this study showed that choline never accumulates at significant level in these mycelia, which were harvested at different culture ages and from the cultures grown under different concentrations of sulfate and phosphate. Instead, significant levels of GB were observed in the mycelia grown in low phosphate medium and its accumulation increased with lowered concentration of sulfate in the medium.

Choline-O-sulfate (COS) accumulation is common among filamentous fungi (Spencer and Harada, 1960, Itahashi, 1961, Spencer et al., 1968, Johnston et al., 1982) and its role in fungal physiology as a sulfur storage molecule was reviewed by Markham et al. (1993). It was reported that COS is likely an endogenous source of choline reserves since the choline-requiring mutants of *Aspergillus nidulans* showed, to an extent, continued growth in the absence of exogenously added choline (Markham and Bainbridge, 1992). However, Arst (1968) argued against COS being the endogenous source of choline, because, even under choline-deficient conditions, *A. nidulans* double mutants, carrying both the *cho*A1 mutation and any mutations defective in sulfate metabolism (*s*-1, *s*-3, *s*-12, or *s*-50 mutations), showed equivalent growth compared to that of the *cho*A1 single mutant. These double mutants can neither synthesize nor utilize COS. This argument led Markham et al. (1993) to suggest that measurable growth of double mutants in choline deficient conditions is due to choline supplied from other alternative sources, other than phospholipids in membranes, of endogenously stored choline. They did not suggest any specific alternative source of endogenous choline. In Chapter 3, it was shown that extracellular phosphocholine-containing pPGM is a precursor of intracellular COS and

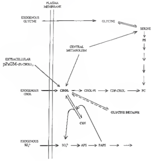
GB. Based on the observation that choline never accumulates at a significant level in the mycelia and that accumulation of GB increases with decrease of COS level, it is suggested that glycine betaine may be another form of endogenous storage of choline in *P. fellutanum* (Fig. 12).

The oxidative conversion of choline to glycine betaine *via* glycine betaine aldehyde (Csonka, 1989, Rhodes and Hanson, 1993) and the interconversion between choline and COS (Harada and Spencer, 1960, Spencer et al., 1968, Gravel, 1976 Markham et al., 1993), are now well established. However, to my knowledge, the reduction of glycine betaine to choline shown by the broken line in Fig. 12, has not been reported. The data reported in Fig. 10 and 11 suggeste that the interconversion between COS and glycine betaine depends on the availability of sulfate and phosphate in the culture medium. Since no significant level of choline was observed in any of these mycelia, whether conversion from glycine betaine to COS is *via* choline as an intermediate metabolite or is a direct conversion process without choline is not clear at present.

Why either glycine betaine or COS, instead of free choline, accumulate at significant level in the mycelia of *P. fellutanum* is not clear. Possibly, the accumulation of glycine betaine and COS prevents a potentiallyl effect of overall positive charge of choline on cellular metabolic processes by substituting the overall neutral charge of glycine betaine and COS for choline. This speculation is supported by the apparent role of glycine betaine or COS as an osmoregulatory compatible solute in bacteria, plants, or animals (Yancey et al., 1982, Csonka, 1989, Rhodes and Hanson, 1993).

Figure 12. Metabolic pathways involving choline which probably occur in *Penicillium fellutanum*.

Based on pathways given in references (Arst, 1968, Gravel, 1976, Markham and Bainbridge, 1992 Markham et al , 1993) and the data in this work. Abbreviations pPGM, peptidophosphogalactomannan, CHOL, choline, COS, choline-O-sulfate; PC phosphatidylcholine PS phosphatidylserine; APS, adenosine-5'-phosphosulfate PAPS 3'-phosphoadenosine-5'-phosphosulfate, CDP, cytidine 5'-diphosphate (This figure is a modification of Fig. 1 in Markham et al , 1993)


PLASMA MEMBRANE
EXOGENOUS GLYCINE
GLYCINE
SERINE
PS
CENTRAL METABOLISM
EXTRACELLULAR pPxGM-(Pi-CHOL)n
EXOGENOUS CHOL
CHOL
CHOL-Pi
CDP-CHOL
PC
GLYCINE BETAINE
COS
EXOGENOUS SO₄²⁻
SO₄²⁻
APS
PAPS

CHAPTER 5
OSMOREGULATION IN PENICILLIUM FELLUTANUM AND A ROLE FOR PHOSPHOCHOLINE-CONTAINING PEPTIDOPHOSPHOGALACTOMANNAN

Introduction

The osmotic potential of the environment is an important physical parameter that influences the ability of microorganisms, algae and plants to grow and survive in a given habitat. Fungi including yeasts are well known for their ability to adapt to environments of high osmolarity by intracellular accumulation of one or more species of low molecular weight compounds to maintain positive turgor pressure (Brown and Simpson, 1972; Brown, 1978; Blomberg and Adler, 1992). These so-called compatible solutes can accumulate to concentrations up to molal levels without greatly interfering with the normal functions of intracellular enzymes; they may also directly protect or stabilize enzymes and other cellular components from high salt concentrations (Brown, 1978; Pollard and Wyn Jones, 1979; Yancey et al., 1982; Arakawa and Timasheff, 1985).

The compatible solutes used by fungi are mainly polyhydroxy alcohols and K. glycerol is the most predominant osmoprotecting polyol (Gustafsson and Norkrans, 1976; Blomberg and Adler, 1992). Other polyols, such as erythritol, arabitol and mannitol, and a disaccharide trehalose, are, though rarely, also osmoresponsive solutes in some filamentous fungi (Jennings, 1984; Gadd et al., 1984; Beever and Laracy, 1986). Oomycetes fungi which are known to be unable to produce polyols, use proline as major compatible solute (Blomberg and Adler, 1992).

Despite the common tolerance of filamentous fungi to very low water activities which is one of the main causes for spoilage of foods (Pitt, 1975), their osmoregulation has rarely been studied *Penicillium* and *Aspergillus* species tested accumulate glycerol as major and erythritol as minor osmoregulatory solute; intracellular accumulation of glycerol increased with increasing concentrations of NaCl and sugars in the culture medium (Adler et al 1982, Hocking and Norton, 1983 Gadd et al , 1984, Beever and Laracy, 1986 Hocking, 1986)

Many organisms including bacteria, algae, plants and animals, use amino acids and their derivatives such as betaines as major osmoregulatory compatible solutes glycine betaine is the most common and predominant osmoprotectant (Le Rudulier et al., 1984, Blunden and Gordon, 1986, Csonka, 1989, Zablocki et al 1991 Rhodes and Hanson, 1993 Galinski, 1995) Choline and phosphocholine are known as osmoprotectants in many bacteria since they are the precursors of the most potent osmoprotectant, glycine betaine (Csonka, 1989 Kaenjak et al., 1993, Galinski, 1995) Glycerophosphocholine (GPC) and glycine betaine are known as osmoprotectants in renal cells of mammals (Zablocki et al., 1991) However, as fungi including yeasts, the role of choline and its derivatives as osmoresponsive compatible solutes has not been extensively studied Choline-O-sulfate, as addition to glycine betaine, is involved in osmoregulation in marine algae, possibly in some bacteria, some marine fungi, and some halophytic plants (Blunden and Gordon, 1986, Hanson and Gage, 1991, Rhodes and Hanson, 1993) However, its role as an osmoprotectant in *Penicillium* and *Aspergillus* species, which are well known as choline-O-sulfate producers (Markham et al , 1993), has not been reported In these organisms, choline-O-sulfate is known as storage molecule of choline and sulfate (Markham et al. 1993).

Filamentous fungi belonging to the genera of *Penicillium* and *Aspergillus* produce extracellular complex polysaccharides termed peptidophosphogalactomannans (pPGMs) (Gander 1974, Leal et al., 1992). Extracellular pPGM of *Penicillium fellutanum* (formerly, *P. charlesii* G. Smith) has as many as 10 α-D-mannopyranosyl 6-phosphocholine phosphodiester residues within the mannan backbone (Unkefer et al. 1982). The major sugars and phosphodiester residues of extracellular pPGM of *P. fellutanum* is shown in Fig. 1 at Chapter 1.

During efforts to determine physiological role(s) of the phosphodiester residues such as phosphocholine in extracellular pPGM, it was demonstrated, in Chapter 3, that this fungus accumulates glycine betaine and choline-O-sulfate inside the mycelia grown under low phosphate condition and that phosphocholine of extracellular pPGM is a precursor of these two intracellular choline derivatives. Furthermore, it was shown that glycine betaine may be another intracellular reserve form of choline under low phosphate and sulfate deficient conditions in *Penicillium fellutanum* (Chapter 4).

Preliminary data showed that this fungus can grow on agar medium containing up to 3M NaCl, which is relatively higher osmolarity condition than the range 0-12% NaCl which defines the salt tolerance levels of *Penicillium chrysogenum* and *Aspergillus niger* (Adler et al., 1982). A series of experiments, therefore, was performed to determine i) how this fungus overcome the external high osmotic stress, ii) whether those intracellular choline derivatives, which at least partly originate from phosphocholine in extracellular pPGM, are involved in this process and thus iii) whether the phosphocholine residues in this polymer are involved in the process of adaptation to high osmotic stress in this fungus.

Materials and Methods

Chemicals, Strain, Media, and Growth

Chemicals and the *P. fellutanum* strain are described in Materials and Methods of Chapter 3. Conidiospore suspensions were prepared as described in Chapter 3, following Beachy's methods (1977), stored at 4°C, and used for routine inoculation. Media compositions are presented in Appendix A. General culture conditions are described in Chapter 3. For salt stress experiments in liquid cultures, varying quantities of sterile solid NaCl were added directly to the culture flasks 72 h after inoculation.

For the stable isotope labeling of methyl-C of choline in extracellular pPGMs and then for examination of intracellular solute pools after the salt stress, 50 mg of L-[*methyl*-^{13}C]methionine dissolved in ddH_2O was added to the culture flasks, using sterile 0.22 µm Millipore membrane filter, 30 h after inoculation.

Preparation of Extracellular Soluble pPGMs

The cultures were harvested on day 8 from either SG or LPSG medium supplemented with or without NaCl; soluble pPGMs were obtained from culture filtrates. The procedures for the isolation and fractionation of soluble pPGMs are described in Materials and Methods of Chapter 3, following the method established by Gander et al. (1974).

^{31}P-NMR Spectroscopy

To examine the compositional changes in pPGMs obtained from the cultures stressed with 3 M NaCl, proton-decoupled ^{31}P-NMR spectrometry was performed as described in Chapter 3 with 150 mg of each sample dissolved in 4 ml ddH$_2$O plus 0.5 ml D$_2$O. The chemical shift, 0.22 ppm, of phosphocholine attached to C-6 position of mannopyranosides in pPGM was used as reference as described in Chapter 3.

Growth of *Penicillium fellutanum* with Salt Stress

To examine the tolerance of *Penicillium fellutanum* to salt stress, spore suspensions were inoculated with the inoculum size of 2.3 mm in diameter on the surfaces of either low phosphate (LPSG) or high phosphate (SG) agar plates containing 0, 0.5, 1.0, 2.0, 3.0, 4.0, or 5.0 M NaCl. The sizes (in diameter) of colonies were measured and compared after 10 days of incubation under constant light at room temperature.

Enzyme assays

Three enzyme activities, nonspecific phosphocholine diester-phosphocholine hydrolase (PC PCH), bis-(p-nitrophenyl)-phosphate phosphodiesterase, (Bis-PDase), and acid phosphomonoesterase, (AP), were determined daily during the incubation of cultures. Enzymes were assayed as described in Chapter 3. For Bis-PDase assay, 2 μmole of bis-(p-nitrophenyl)-phosphate was used as substrate.

Preparation of Mycelial Extracts

The intracellular solute pools of mycelia obtained from salt-stressed LPSG cultures enriched with L-[methyl-^{13}C]methionine and supplemented with varying concentrations of NaCl (0 5, 1 0, 2 0 or 3 0 M), were examined after extraction of mycelia with either perchloric acid or 80% ethanol Each culture was harvested on day 8 which was one or two days after maximum PC PCH activity was attained in the cultures supplemented with greater than 1 M NaCl This time (harvest day) was selected to give enough time for extracellular PC PCH to cleave off the phosphocholine residues from the extracellular pPGMs so that either phosphocholine or choline can be taken up into the cell, and yet insufficient time for intracellularly accumulated compounds to disappear by further metabolism or possible leakage from the cell Mycelia were obtained by filtration *in vacuo* using either C- or M-type sintered glass filters and by washing with ddH_2O containing the same salt concentrations as the parent culture to avoid any possible loss of intracellular solutes during washing of harvested mycelia.

Initially, perchloric acid extraction was performed The mycelia obtained from 200 ml of each culture were treated with 10-20 ml of 3 M perchloric acid for 5 h at 4°C. The pH of extracts was neutralized by adding solid $KHCO_3$ and then allowed to stand overnight at 4°C The precipitate was removed by centrifugation at 9,000 x g for 30 min and then filtered in vacuo through M-type sintered glass filter The possibility of acid hydrolysis of phosphoester bond and sulfate ester bond in any intracellular solutes (such as phosphocholine or choline-O-sulfate) during 3 M perchloric acid treatment of mycelia, was examined, the same reference compounds were treated in the same way and analyzed by either ^{31}P- or ^{13}C-NMR spectrometry The spectra showed no differences in chemical shifts, indicating that these compounds are stable in 3 M perchloric acid for at least 5 h. However, the extracts obtained by perchloric acid treatment contained a significant quantity of carbonate which interfered with the data acquisition of other compounds by creating a dynamic range problem during ^{13}C-NMR spectrometry. Throughout this research, 80% ethanol extraction, as described in Materials and Methods of Chapter 3 was performed and found to be less laborious than perchloric acid extraction.

Detection of Osmoregulatory Compatible Solutes by ^{13}C-NMR Spectroscopy

Penicillium fellutanum was grown in 200 ml of low phosphate medium with the indicated concentrations of NaCl (0.5, 1.0, 2.0, or 3.0 M) and the mycelia were harvested on day 8. The cultures were also enriched with L-[*methyl*-^{13}C]methionine to make the detection of any methyl carbon containing intracellular solutes easier. Each sample which contains 4 ml of mycelial extract, 0.5 ml of D_2O and 0.75% TSP as internal reference, was analyzed by proton-decoupled ^{13}C-NMR spectroscopy. The spectra were recorded at 75 MHz as described in Materials and Methods of Chapter 3. Frequencies and acquisition parameters are described in the legends of each Figure.

Osmotic Downshock

To determine whether the increased accumulation of intracellular solutes (glycerol, glycine betaine, and choline-O-sulfate) was to cope with the external high osmolarity, the mycelia grown for 8 days in low phosphate LPSG medium stressed with 3 M NaCl were harvested and transferred asceptically to a fresh LPSG medium without added NaCl. The transferred mycelia were harvested 3 days after transfer and was analyzed with ^{13}C-NMR spectrometry after ethanol extraction. To more clearly monitor the fate of these intracellular solutes, the initial salt-stressed mycelia (before transfer) were grown with L-[*methyl*-^{13}C]methionine as described before.

Identification of Intracellular Osmoregulatory Polyols

The polyols in the intracellular solute pools were further identified by paper chromatography. Ten microliters of each cell extract obtained from the cultures stressed with varying concentrations of NaCl (0.5, 1.0, 2.0, or 3.0 M) were spotted on Whatman No. 3 paper. Ten µl of each reference polyol (glycerol and erythritol;100 mM each) was also spotted. The chromatogram was developed in a descending manner with *n*-butanol:pyridine:ddH$_2$O (6:4:3, w/v/v) for 11 h at room temperature in a glass tank lined with the same paper previously saturated with the solvent employed. The bottom edge of the chromatogram was serrated, and the solvent was allowed to drip off the chromatogram. The chromatogram was then taken out of the developing tank, dried and soaked into the first staining solution (mixture of 2 ml of 40% AgNO$_3$ and 80 ml of acetone). After air drying, it was treated with a mixture of 10 ml of 10 N NaOH and 90 ml of ethanol. The wet chromatogram was finally heat dried for 5 min in 100°C drying oven, fixed with 0.2 M Na$_2$S$_2$O$_3$, and then air dried in the hood.

Growth Inhibition by NaCl and Role of Exogenous Choline and Sulfate

Penicillium fellutanum was cultured in low phosphate medium (20 ml per flask) stressed with 2 M NaCl. Sterile solid NaCl was added directly 72 h after inoculation and either choline chloride (final concentration, 2 mM) or Na$_2$SO$_4$ (final concentration, 10 mM) was added to the cultures 1 h after the addition of NaCl Choline chloride and Na_2SO_4 were dissolved in ddH_2O and added to the culture after filter-sterilized using sterile 0 22 µm Millipore membrane Growth was determined by measuring the mycelial dry weight as described in Chapter 4 The cultures were taken at time intervals and filtered *in vacuo* through pre-weighed Whatman No 1 filter paper on a sintered glass filter and then dried at 100°C for 5 h.

The effect of exogenously added choline on the osmotolerance of this fungus was also examined by measuring the radial growth of the colony on the C source limiting, low phosphate culture plates supplemented with 3 M NaCl To reduce the accumulation of polyols and stimulate the accumulation of other osmoregulatory compatible solutes such as choline derivatives, only one tenth of the C-source (glucose) was used for these culture plates The medium was supplemented with 2 mM choline chloride and spore suspension was inoculated on the surface of the plates with a diameter of 2 3 cm. The diameter of the colonies was measured daily for 29 days

Quantification of Intracellular Compatible Solutes

The amounts of glycine betaine and choline-O sulfate were determined by ^{13}C-NMR spectrometry using TSP as reference as described in Materials and Methods in Chapter 4 Each signal was integrated using Felix for Windows 1 02 software (Molecular Simulations Inc) and the integrated values were compared with that of TSP

Results

Osmotolerance of *Penicillium fellutanum*

The conidiospores of *P. fellutanum* inoculated on the surface of either low phosphate LPSG (Fig. 13A) or high phosphate SG agar plates (Fig. 13B) containing different concentrations of NaCl, were able to germinate and grow on plates containing up to 3 M NaCl (Table 3). In both low phosphate and high phosphate media, the size of colonies decreased with increasing concentration of NaCl. There was no germination and no growth on the plates containing over 4 M NaCl. Although there was no pigmentation and no hyphal growth on the plates containing 2 M and 3 M NaCl, they germinated and grew with slime mold-like colony morphology. The colonies grown in low phosphate LPSG medium (Fig. 13A) showed less growth than those in high phosphate SG medium (Fig. 13B) at up to 0.5 M NaCl, however, they showed more growth with greater than 2 M NaCl. The data suggest that the fungus shows more tolerance to high salt stress when it is grown in low phosphate conditions than in high phosphate conditions.

Production of Phosphoesterases in Low Phosphate Medium with NaCl

Over one to four days after adding NaCl ranging from 0.5 M to 3 M to the LPSG medium, the production of a nonspecific phosphocholine diester: phosphocholine hydrolase (PC:PCH) remained constant up to 2 M NaCl and decreased slightly in the culture containing 3 M NaCl, although it had a narrower range of production compared with that of control culture without NaCl (Fig. 14). Whereas, those of bis-(p-nitrophenyl)-phosphate phosphodiesterase (Bis-PDase) and an acid phosphomonoesterase

(AP) were inhibited significantly as the salt concentrations increased Surprisingly the PC PCH was produced for much longer period of time in 2 M or 3 M NaCl-containing medium than in 0 5 or 1 0 M NaCl, indicating that the activity of PC PCH may have an important role when *P fellutanum* is grown under low phosphate and high osmolarity conditions.

DEAE-Cellulose Column Chromatography of pPGMs

The crude pPGMs preparations obtained from LPSG medium stressed with 3 M NaCl and the pPGMs from unstressed control cultures were fractionated through DEAE-cellulose (DE-52) anion exchange column. Fig 15A shows the elution pattern of control pPGMs isolated from unstressed culture medium, ~70% of the polymers (peak I) elutes with 0 01 N HCl/0 06 M LiCl and ~30% of the polymers (peak II) elutes with 0 01 N HCl/0 4 M LiCl However the salt-stressed pPGMs showed that the minor portion (~30% of the polymers, peak I) came out first and the major (~50% peak II) came out later (Fig 15B) This change in elution patterns from DE-52 column suggests that the compositions of pPGMs changed with 3 M NaCl

^{31}P-NMR Spectroscopy of pPGMs

The spectrum in Fig. 16A represents the pPGM obtained from low phosphate LPSG medium without NaCl added (peak fraction I in Fig. 15A) The spectrum in Fig. 16B represents the salt-stressed pPGM obtained from LPSG medium containing 3 M NaCl (peak fraction I in Fig 15B)

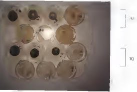


Figure 13. Osmotolerance of *Penicillium fellutanum* to NaCl.

The mycelia were grown on the plates of A) high phosphate SG medium or B) low phosphate LPSG medium containing 0, 0.5, 1, 2, 3, 4, or 5 M NaCl. The set of plates shown represents one set from the 3 replicate sets. For details, see the legend of Table 3.

Table 3 Osmotolerance of *Penicillium fellutanum* to NaCl

	NaCl (M)							Inoculum size (cm)[a]	PC PCH[*]
	control[b]	0.5	1.0	2.0	3.0	4.0	5.0		
LPSG[c]	4.8 cm	4.0	3.7	2.5	1.7	_	_	2.3	+
SG[d]	4.9 cm	4.4	3.7	2.4	1.2	_	_	2.3	_
pigment	Y	Y	Y	N	N	NA	NA		
hyphae	Y	Y	Y	N	N	NA	NA		

[a] Spore suspensions were inoculated with a inoculum size of 2.3 cm in diameter as a single spot per plate on the surfaces of both LPSG and SG agar plates containing different concentrations of NaCl up to 5 M. Cultures were grown for 10 days and the diameter of each colony was measured and compared. Culture conditions are described in Materials and Methods. [b] Control plates contained 20 mM NaCl. [c] LPSG: low phosphate standard growth medium containing 2 mM Na_2HPO_4. [d] SG: high phosphate standard growth medium containing 20 mM Na_2HPO_4. "Y" stands for Yes, "N" for No, and "NA" for Not applicable. [*] The activity of PC PCH, (non-specific phosphocholine diester phosphocholine hydrolase), was detected in liquid cultures (see Fig. 14). Each value represents the mean of triplicates.

Figure 14. **Production of phosphoesterases in low phosphate medium containing NaCl.**

Seventy two hours after the inoculation of spore suspension into LPSG medium, sterile solid NaCl 0 0.5 1.0 2.0 3.0 M in final concentrations (A through E panels, respectively) was added to each culture flask. Three enzymes, nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH), and phosphomonoesterase (AP) and bis-(p-nitrophenyl)-phosphate phosphodiesterase (Bis-PDase) were assayed daily during 15 day long culture. Two to three milliliters of culture broth were taken and filtered through Whatman No. 4 paper and immediately assayed for enzyme activities as described in Materials and Methods.

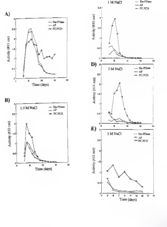
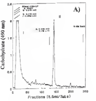
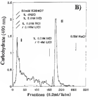

A)
Bis-PDase
AP
PC:PCH
Activity (410 nm)
Time (days)
B)
0.5 M NaCl
Bis-PDase
AP
PC:PCH
Activity (410 nm)
Time (days)
C)
1 M NaCl
Bis-PDase
AP
PC:PCH
Activity (410 nm)
D)
2 M NaCl
Bis-PDase
AP
PC:PCH
Activity (410 nm)
E)
3 M NaCl
Bis-PDase
AP
PC:PCH
Activity (410 nm)
Time (days)

Figure 15. DEAE-Cellulose column chromatography of extracellular peptido-phosphogalactomannans (pPGMs).

The culture supernatants obtained from low phosphate LPSG medium grown with 3 M NaCl and without NaCl as control, were used for the isolation and purification of pPGMs. Fractions were assayed for the total carbohydrate with 0.242 mM glucose solution as a reference. A) panel is the elution pattern of control pPGM obtained from LPSG medium without NaCl and B) panel is that of the salt-stressed pPGM obtained from LPSG medium containing 3 M NaCl. The O.D. values of over 2.0 are infinitive values. After fractionation of pPGMs on DEAE-cellulose, each peak fraction was lyophilized separately and applied to ^{31}P NMR analysis (see Fig. 16).


A)
Carbohydrate (490 nm)
Fractions (5.5ml/Tube)
I
II
0
50
100
150
200
250


B)
50mM K2B4O7
1L dH2O
1L 0.01N HCl
1L 0.01N HCl
/ 0.08M LiCl
1L 0.01N HCl
/ 0.4M LiCl
0.5M NaCl
I
II
Carbohydrate (490 nm)
Fractions (5.2ml/Tube)
0
0.5
1
1.5
2
2.5
0
50
100
150
200
250
300

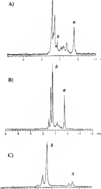
The spectrum in Fig. 16C represents the pPGM obtained from peak fraction II in Fig. 15B. It was previously shown that the signal intensity of phosphocholine (at 0.22 ppm) decreased in pPGM isolated from low phosphate LPSG medium (Fig. 16A) when compared with the pPGM isolated from high phosphate SG medium (see Fig. 4 in Chapter 3). As shown in Fig. 16B and 16C, this signal was further decreased in pPGM isolated from LPSG medium stressed with 3 M NaCl. The ratios of the signal intensities between phosphocholine (designated as *a* at 0.22 ppm) and a signal at 1.1 ppm (designated as *b*), which is arbitrarily selected for comparison of signal intensities, significantly decreased in pPGM from salt-stressed cultures when compared with the ratios of the normal pPGM (isolated from the medium without added NaCl). These results suggest that phosphocholine released from pPGM may have an important role during the growth in high osmolarity conditions, while also serving as a reserve source of phosphate.

^{13}C-NMR Analysis of Intracellular Solute Pools

It was previously shown that the mycelia grown in low phosphate LPSG medium accumulate glycine betaine and choline-O-sulfate which originate from phosphocholine released from extracellular pPGM by PC PCH activity (Chapter 3). To determine whether the accumulation of these intracellular choline derivatives is sensitive to external osmolarity, intracellular solute pools of mycelia grown for 8 days in low phosphate LPSG medium containing varying concentration of NaCl, were examined after 80% ethanol extraction. As it was shown that ^{13}C from L-[*methyl*-^{13}C]methionine is incorporated *in vivo* into methyl groups of mannosyl-6-phosphocholine in extracellular pPGMs (Fig. 5B in Chapter 3), all cultures were enriched with L-[*methyl*-^{13}C]methionine as described before

Figure 16 Proton-decoupled ^{31}P-NMR spectra of the pPGMs isolated from low phosphate medium stressed with 3 M NaCl.

A) spectrum is the control pPGM which is the fraction I in Fig. 15A, and B) and C) are the fraction I and II obtained from DE-52 column in Fig. 15B One hundred fifty milligrams of each pPGM were dissolved in ddH$_2$O with 10% D$_2$O Data acquisition parameters were, for control pPGM, spectral width, 2 403 8 Hz (8 064 5 Hz) pulse width, 21 µs (18 µs), acquisition time, 1 70 s (507 9 ms), spectrometer frequency, 121 4685 MHz (121 4687 MHz), temperature, 23°C (8°C) Numbers in brackets are for pPGMs in B) and C) Chemical shifts were previously assigned by Unkefer et al (1982) *a* and *b* signals represent phosphocholine attacted to C-6 position of mannosyl residum and *N*-peptidyl-phosphoethanolamine attached to C-6 position of galactofuranosyl residues, respectively


A)
a
b
3
2
1
0
-1
PPM
B)
b
a
5
4
3
2
1
0
-1
-2
PPM
C)
b
a
2.5
2.0
1.5
1.0
0.5
0.0
-0.5
PPM

Glycine Betaine and Choline-O-Sulfite Are Osmoprotectants in *P. fellutanum*

The major intracellular methyl amine solutes in *P. fellutanum* grown for 8 days in low phosphate LPSG medium with 3 M NaCl, were choline-O-sulfate and glycine betaine (Fig. 17A). The chemical shifts for choline-O-sulfate (COS) and glycine betaine (GB) were previously assigned (see Fig. 7 and 8 in chapter 3), signal at 56.76 ppm represents -N$^+$-(CH$_3$)$_3$ of choline-O-sulfate and signal at 56.23 ppm represents -N$^+$-(CH$_3$)$_3$ of glycine betaine. The two signals upfield at 54.99 and 48.82 ppm were also considered as methylamine compounds and, however, were not of concern since they did not change with increasing concentration of NaCl. There were no significant differences in the pattern and intensities of signals on ^{13}C-NMR spectra of the intracellular solute pools of those mycelia grown with up to 1 M NaCl (Fig. 17C, 17D and 17E). As shown in Fig. 17E, choline-O-sulfate (designated as *COS*1) and glycine betaine (designated as *GB*1) accumulated in the mycelia grown in low phosphate culture medium without NaCl added (see also Chapter 3). This suggests that choline-O-sulfate and glycine betaine are the so-called constitutive compatible solutes (in accordance with the terminology of Harris, 1981) in *P. fellutanum* growing under low phosphate conditions. However, the accumulation of glycine betaine significantly increased in the mycelia grown with over 1 M NaCl (Fig. 17A and 17B), suggesting that glycine betaine is an osmoregulatory compatible solute in *P. fellutanum* growing under high osmolarity conditions. This conclusion was further strengthened by the observation that the levels of glycine betaine accumulated under high osmolarity conditions significantly decreased when the salt stress was removed (Fig. 18B). The amounts of glycine betaine and COS were determined by integrating the individual signal on ^{13}C-NMR spectrum and comparing the values obtained to that of reference TSP (Table 4) The amount of glycine betaine increased 22-fold upon 3 M NaCl stress. These results clearly showed that glycine betaine (and thus choline) which originated from phosphocholine attached to extracellular pPGM is involved in osmoregulation in *Penicillium fellutanum* under low phosphate and high osmolarity conditions Whether choline-O sulfate is involved in osmoregulation in this fungus is not clear Unlike glycine betaine there was a small increase in the signal intensity of COS in the mycelia stressed with up to 3 M NaCl (Fig 17) However, the mycelia grown in low phosphate medium with 3 M NaCl for 8 days accumulated about 2 6-fold increased COS (Table 4) This suggests that COS may also be involved in osmoregulation in this fungus.

Glycerol and Erythritol Are the Major Osmoregulatory Polyols in *P. fellutanum*

The two signals which resonated downfield at 65 47 and 74 95 ppm in Fig 17 represent -CH$_2$OH and -CHOH, respectively, of either glycerol or erythritol Glycerol and erythritol were identified by ^{13}C-NMR spectroscopy and paper chromatography (see below; Fig. 19 and 20), the chemical shifts of these two signals exactly matched with those of C1,3, (-CH$_2$OH), and C2 (-CHOH-) from glycerol and C1,4, and C2,3 from erythritol (Fig. 19)

Hocking and Norton (1983) argued that glycerol cannot be distinguished from erythritol since C1,4 and C2,3 from erythritol show chemical shifts too close to those of C1,3 and C2, respectively, from glycerol However the ratio of signal intensities for C1,4 C2,3 of erythritol should be same, while C1,3 C2 for glycerol should be 2-fold. In the mycelia grown with 0 to 1 M NaCl (Fig. 17C, 17D and 17E), it appears that there was mainly erythritol based on the ratio of intensities of two signals (65.47 to 74.95 ppm). This was consistent with the result from paper chromatography (Fig. 20) which showed an erythritol but no significant spot equivalent to glycerol in the mycelia grown without NaCl added (Fig. 17E and lane 1 in Fig. 20), although the chromatogram (lane 6 and 7 in Fig. 20) showed that the mycelia grown with 1 and 0.5 M NaCl (Fig. 17C and 17D, respectively) contained slightly more erythritol than the mycelia grown without NaCl added (Fig. 17E and lane 1 in Fig. 20). However, as the concentration of NaCl increased to over 2 M (Fig. 17A and 17B), the ratio of signal intensities of two peaks (65.47 to 74.95 ppm) changed and became almost 2.00, indicating that as the concentration of NaCl increased, erythritol disappeared and glycerol became the major polyol compound in higher osmolarity conditions (at 3 M NaCl). This was also confirmed through paper chromatography (lane 2 and 5 of Fig. 20). Thus, Fig. 17A shows that glycerol was the only major polyol in *P. fellutanum* grown in 3 M NaCl-containing low phosphate LPSG medium. However, the mycelia grown with 2 M NaCl (Fig. 17B and lane 5 in Fig. 20) used glycerol and erythritol as major polyols for osmotic balancing. Thus, erythritol is the major constitutive compatible solute which presents in mycelia grown without osmotic stress. These osmolytes enable *P. fellutanum* to tolerate up to 1 M NaCl; however, at 2 M or 3 M NaCl, glycerol accumulation becomes significant and erythritol starts to disappear and finally, glycerol becomes the only major osmoregulatory polyol in the mycelia stressed with 3 M NaCl.

Figure 17. ^{13}**C-NMR spectra of the intracellular solutes in *Penicillium fellutanum* grown with varying concentrations of NaCl.**

Cultures were stressed with varying concentration of NaCl (0, 0.5, 1.0, 2.0, or 3.0 M) by adding sterile solid NaCl directly into each 200 ml culture flask 72 h after inoculation. All cultures were harvested on day 8. To increase the sensitivity of all the methyl-amine containing intracellular solute, all cultures were enriched with 50 mg L-[*methyl*-^{13}C]methionine per flask. After ethanol extraction and drying *in vacuo*, the residues were dissolved in 4 ml ddH$_2$O and water soluble portion was analyzed with ^{13}C-NMR spectrometry. To 4 ml of each extract, 0.5 ml D$_2$O containing 0.75% TSP was added as internal reference. Data acquisition parameters were, for all samples: spectral width, 10,000 Hz; pulse width, 28 µs; acquisition time, 409.6 ms; spectrometer frequency, 75.457 MHz; scans, 1,000; temperature, 10°C. For simplicity, only 40 to 80 ppm regions are shown in the spectrum. Symbols: *COS*1 represents -N$^+$-(CH$_3$)$_3$ of choline-O-sulfate (COS); *GB*1 represents -N$^+$-(CH$_3$)$_3$ of glycine betaine; G, glycerol; E, erythritol. For chemical shifts assignment for COS and GB, see Table 1 in Chapter 3.

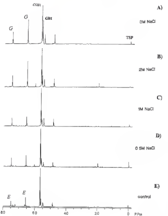

COS1
G
G
GB1
A)
3M NaCl
TSP
B)
2M NaCl
C)
1M NaCl
D)
0.5M NaCl
E)
E
E
control
80
60
40
20
0
PPM

Table 4 Increased accumulation of glycine betaine and choline-O-sulfate in *Penicillium fellutanum* by high osmotic stress

	LPSG medium		
Intracellular solutes	Control	(+) 3.0 M NaCl	Fold increase
COS (mg)	9.3	25.0	2.7
GB (mg)	1.5	33.2	21.9
Growth (g D.W.)	3.5 ± 0.35	1.8 ± 0.01	

The mycelia were grown for 8 days in 200 ml each of low phosphate LPSG medium in three separate flasks with 3 M NaCl and without NaCl added. The mycelia were harvested and applied to ^{13}C NMR analysis after ethanol extraction as described before. TSP was used as reference and the data were acquired without NOE enhancement. The amount of each solute was determined by integrating signals on ^{13}C-NMR spectra using Felix for Windows 1.02 software (Molecular Simulations Inc.). The integrated values were compared with that of TSP. The mycelial dry weight was determined by taking the mean value of triplicate samples as described before in Chapter 4. Symbols: COS, choline-O-sulfate; GB, glycine betaine.

Figure 18. Influence of osmotic downshock on the levels of intracellular solutes.

To determine whether the increased accumulation of intracellular solutes (glycerol, glycine betaine, and choline-O-sulfate) was to cope with the external high osmolarity (2 M or 3 M NaCl in Fig. 17A and 17B), the mycelia grown for 8 days in low phosphate LPSG medium stressed with 3 M NaCl were harvested and transferred aseptically to a fresh LPSG medium containing A) 3 M NaCl as control and B) without NaCl. The transferred mycelia were harvested 3 days after transferred and analyzed with ^{13}C-NMR spectrometry after ethanol extraction. To more clearly monitor the fate of those intracellular solutes, C) the initial salt-stressed mycelia (before transfer) were grown with L-[*methyl*-^{13}C]methionine as described before. Acquisition parameters were same as described in Fig. 17.

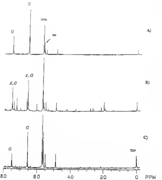

G
G
om
A)
E,G
E,G
B)
G
G
TSP
C)
80
60
40
20
0
PPM

Figure 19 Identification of intracellular glycerol and erythritol.

The signals at 65.4 and 74.9 ppm on ^{13}C-NMR spectrum of the mycelial extracts obtained from the cultures stressed with 3 M NaCl were partially identified by comparing the chemical shifts with those of suspected chemicals (glycerol and erythritol) dissolved in ddH_2O. A) 500 mM glycerol and B) 100 mM erythritol. The spectrum C) was obtained with the extract of mycelia grown for 8 days with 3 M NaCl in 200 ml of low phosphate medium (see Fig. 17A also). To 4 ml of each sample, 0.5 ml D_2O containing 0.75% TSP was added as internal reference. Scans were A) 344, B) 1,160, C) 1,146, respectively. Data acquisition parameters were, for all samples, spectral width, 10,000 Hz; pulse width, 28 µs, acquisition time, 409.6 ms, spectrometer frequency, 75.457 MHz, temperature, 10°C. Complete identification was achieved by paper chromatography (see Fig. 20).

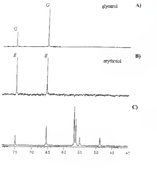

G
G
glycerol
A)
E
E
erythritol
B)
C)
75
70
65
60
55
50
45
40

Figure 20. Paper chromatographic separation and identification of glycerol and erythritol.

The mycelial extracts were obtained from LPSG medium stressed with different concentration of NaCl, 0 (lane 1), 0.5 (lane 7), 1.0 (lane 6), 2.0 (lane 5), 3.0 M (lane 2). Ten µl of each cell extracts was applied to Whatman No. 3 paper with 10 µl of each reference solute: 20 mM glycerol (lane 3), 20 mM erythritol (lane 4). Procedures are described in Materials and Methods. Symbols: ctl*, mycelial extracts obtained from the cultures without added NaCl as control, Gly, glycerol, Ery, erythritol.

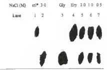

NaCl (M)
ctl*
3.0
Gly
Ery
2.0
1.0
0.5
Lane
1
2
3
4
5
6
7

Table 5 Rf values of polyols on paper chromatogram of the mycelial extracts of *Penicillium fellutanum*

	Rfs	
extracts*	glycerol	erythritol
	1.00	0.81
control	ND	0.78
0.5 M	0.97	0.74
1.0 M	0.97	0.74
2.0 M	1.00	0.78
3.0 M	1.00	faint

Rf values were calculated with the value of glycerol which moved the furthest, set at 1.00

* Each extract represents the mycelial extract obtained from low phosphate LPSG culture medium after the growth for 8 days with 0.5, 1.0, 2.0 or 3.0 M NaCl, respectively; control, without NaCl added

The role of glycerol in osmoregulation was further confirmed by the observation that the level of glycerol accumulated in the mycelia by 3 M NaCl stress significantly decreased upon removal of salt stress by transferring the salt-stressed mycelia (Fig. 18B) to a fresh LPSG medium without NaCl added (Fig. 18A).

Exogenous Choline and Sulfate Enhance Osmotolerance of *P. fellutanum*

Penicillium fellutanum was grown in low phosphate LPSG medium stressed with 2 M NaCl. NaCl was added 72 h after inoculation. Either 2 mM choline chloride or 10 mM Na_2SO_4 was added 30 min after addition of NaCl and the growth was measured by dry weight. Neither choline nor sulfate stimulated the growth of unstressed mycelia in low phosphate LPSG medium (Fig. 21A). However, addition of 2 mM choline stimulated the growth 1.7-fold in total dry weight when measured 3 days after addition of choline to salt stressed cultures (Fig. 21B). Growth stimulation may have been due to enhanced accumulation of glycine betaine (GB) in mycelia grown under high osmolarity conditions. Increased GB accumulation was confirmed by ^{13}C-NMR spectroscopy of the mycelial extracts (not shown). The addition of 10 mM sulfate in the same manner stimulated the growth about 2.5-fold in total dry weight after a lag period of about 2 days (Fig. 21B).

Enhanced osmotolerance by exogenously added choline was also observed in the cells grown on agar plates stressed with 3 M NaCl (Fig. 22). The low phosphate agar medium contained 10-fold less glucose than usual low phosphate medium, this was to decrease the synthesis of osmoregulatory polyols such as glycerol and thereby to increase the possibility of synthesis of other compatible solutes such as glycine betaine and choline-O-sulfate. The spore suspension was inoculated in a diameter of 2.7 cm on the surface of agar plate containing 2 mM choline chloride and allowed to grow up to 29 days. The control plate without choline added has a faint smear with much smaller diameter (~ 0.7 cm) of growth than the diameter of the colony on the plates with 2 mM choline added, it appeared that the spores, or the mycelia right after the germination, on the control plates were being lysed during 29-days of incubation under low phosphate, C-source limiting, and 3 M NaCl-stressed conditions. However, although there was no hyphal growth, the colony on the plate containing 2 mM choline was significant with a diameter about the same size as the inoculum size, this suggested that the spores on the plates containing 2 mM choline could germinate and survive the harsh environmental conditions better than those spores inoculated on the control plates. These results suggest that choline is also an osmoprotectant in *P. fellutanum* in that it is a precursor of intracellular osmoprotectant, glycine betaine (GB) and/or choline-O-sulfate (COS).

Discussion

Osmoregulation in *P. fellutanum* and Involvement of Phosphocholine of pPGM

Glycine betaine and its oxidation precursor choline are known to be the most predominant osmoregulatory compatible solutes in a wide variety of organisms including bacteria (Csonka, 1989; Galinski, 1995), marine algae (Blunden and Gordon, 1986), higher plants (Rhodes and Hanson, 1993), and animals (Zablocki et al., 1991). However, the presence of choline derivatives such as choline, glycine betaine, and choline-O-sulfate as osmoregulatory compatible solutes has not been reported in yeasts and filamentous fungi belonging to the genera of *Penicillium* and *Aspergillus*. Instead, polyhydroxy alcohol compounds such as glycerol, arabinitol, erythritol are the most predominant compatible solutes in fungi including yeasts (Blomberg and Adler, 1992).

It was demonstrated (in Chapters 3 and 4) that *Penicillium fellutanum* grown under low phosphate condition in the culture medium accumulates glycine betaine and choline-O-sulfate in the mycelia, and that these two intracellular solutes originate from the phosphocholine attached to the extracellular peptidophosphogalactomannan (pPGM). A nonspecific phosphocholine diester-phosphocholine hydrolase (PC PCH) is known to catalyze the release of phosphocholine from extracellular pPGM (Salt and Gander, 1988).

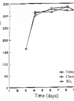
During efforts to determine the physiological role of phosphocholine released from pPGM in *Penicillium fellutanum*, a series of experiments was performed to determine if this fungus uses any choline derivatives for its osmoregulatory processes and if extracellular pPGM is involved in these processes.

It was shown that *P. fellutanum* can grow in a medium containing up to 3 M NaCl, this indicates that it is one of the halotolerant and xerotolerant fungi. The data show that *P. fellutanum* uses glycine betaine, choline-O-sulfate, glycerol, and erythritol as osmoregulatory compatible solutes when grown for 8 days under low phosphate and high osmolarity conditions. Glycerol, glycine betaine, and choline-O-sulfate were the major osmoregulatory solutes at 3 M NaCl. The major compatible solutes at 2 M NaCl were glycerol, glycine betaine, choline-O-sulfate, and erythritol. Erythritol and choline-O-sulfate were the major ones at up to 1 M NaCl.

Figure 21 Enhanced osmotolerance of *Penicillium fellutanum* upon addition of choline and sulfate.

The spore suspension (0.3 ml) of *P. fellutanum* was inoculated into each flask containing 20 ml of low phosphate LPSG medium A) without or B) with 2 M NaCl. Seventy two hours after inoculation, sterile solid NaCl was added to the final concentration of 2 M and 1 h later, either sterile choline chloride solution in ddH_2O (final concentration 2 mM) or Na_2SO_4 (final concentration 10 mM) was added to each salt stressed culture. Growth was determined by taking the mean values of duplicate mycelial dry weight.

A)


350
300
250
200
150
100
50
0
1
2
3
4
5
6
7
8
9
10
Growth (D W in mg)
Time (days)
Control
Choline
SO₄

B)

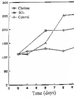
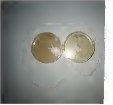

300
250
200
150
100
50
0
2
3
4
5
6
7
8
9
10
Growth (D W in mg)
Time (days)
Choline
SO₄
Control

Figure 22. Enhanced osmotolerance of *Penicillium fellutanum* by exogenous choline.

The spore suspension of *P. fellutanum* was inoculated as a single spot with a diameter 2.7 cm on to the center of the surface of low phosphate LPSG agar plates containing 3 M NaCl and 2 mM choline chloride. The radial growth was measured daily up to 29 days and compared with the control plates without choline chloride. The set of plates shown represents one set from the 3 replicate sets.



A) + choline (2 mM) B) control (- choline)

The level of COS increased only 2.6-fold upon 3 M NaCl stress; however, that of GB increased 22-fold (Table 4), suggesting that this fungus preferentially use GB as osmoprotectant. However, it should be noted that, as shown in Chapter 4, the accumulation of COS depends on the availability of sulfate in the culture medium. Thus, with sufficient sulfate in the culture medium, role of COS as an osmoregulatory compatible solute would become more significant.

Glycine betaine and choline-O-sulfate as well as erythritol were found in the mycelia grown in low phosphate medium without NaCl added (see Chapter 3 also) and the level of these solutes increased with salt stress, indicating that these solutes are constitutive compatible solutes under low phosphate conditions (Harris, 1981).

The addition of choline chloride (2 mM) to the cultures stressed with 2 M or 3 M NaCl stimulated the growth of *P. fellutanum* (Fig. 21 and 22). This result suggests that choline is also an osmoprotectant in this fungus in that it is a precursor of intracellular glycine betaine and COS.

The addition of sulfate (10 mM) to the cultures containing 2 M NaCl stimulated the growth by up to 2.5-fold in total dry weight (Fig. 21). This was probably due to enhanced synthesis of COS with sufficient sulfate, resulting in enhanced osmotolerance. The increased accumulation of COS was previously demonstrated in the mycelia grown in low phosphate medium (Fig. 10 and 11 in Chapter 4).

The ^{13}C-NMR analysis of intracellular solutes of the mycelia grown for 8 days on low phosphate medium with concentrations of NaCl ranging from 0 to 3.0 M, showed that glycine betaine and glycerol accumulation started to increase in the mycelia grown in low phosphate medium stressed with 2 M NaCl as shown in Fig. 17. The colonies grown on the low phosphate agar medium showed less growth than the colonies on the high phosphate agar medium. However, at 2 M and 3 M NaCl, they showed more growth on the low phospahte agar plates than those on the high phosphate plates (Fig. 13). This suggests that *P. fellutanum*, when it grows in low phosphate conditions, shows more tolerance to high osmotic stress (2 M and 3 M NaCl) than to low osmotic stress (0 to 1.0 M NaCl).

Three phosphoesterases are produced at high levels during the growth of *P. fellutanum* in low phosphate (2 mM phosphate) medium: a nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH), an acid phosphomonoesterase (AP), and an unknown bis-(p-nitrophenyl)-phosphate specific phosphodiesterase (Bis-PDase) (Fig. 14). These three enzymes are probably the phosphate scavengers.

During the growth of *P. fellutanum* in low phosphate medium containing up to 3 M NaCl (Fig. 14), a constant, high level production of PC PCH was observed, although it showed a narrower range of production compared with that of control culture without NaCl, the production of the other two enzymes (Bis-PDase and AP) was significantly inhibited. Interestingly, PC PCH was produced for a longer period of time in the cultures stressed with 2 M or 3 M NaCl compared with the cultures stressed with 0.5 or 1 M NaCl. It appears that high osmotic stress (2 M or 3 M NaCl) stimulates the production of this enzyme. The data also suggest that, at NaCl concentration up to 1 M, the PC PCH activity is less critical for overcoming salt stress but, at concentration of 2 or 3 M NaCl, the activity of this enzyme becomes more important for the cells to overcome the higher salt stress.

In previous chapters, it was shown that intracellular glycine betaine and choline-O-sulfate originate from phosphocholine in pPGM (Chapter 3), and that mycelia grown in 2 mM phosphate medium accumulate glycine betaine and 1 5 fold more choline-O-sulfate than the mycelia grown in 20 mM phosphate medium (Chapter 4) This correlates with high level production of PC PCH in 2 mM phosphate medium

The ^{31}P-NMR spectra of the pPGMs isolated from 3 M NaCl-stressed cultures showed a significantly decreased level of phosphocholine residues (Fig 16) The ratios of the signal intensities between phosphocholine (resonating at 0 22 ppm) and a signal at 1 1 ppm, which is arbitrarily selected for comparison of signal intensities, significantly decreased in salt-stressed pPGMs when compared with the ratios of the normal pPGM isolated from the low phosphate medium without NaCl added This suggests that, under 3 M NaCl stress phosphocholine residues were preferentially released from extracellular pPGMs, this result correlates with the relatively high level of PC PCH activity in the medium containing 2 M or 3 M NaCl, when compared with the levels of other two phosphoesterases, Bis-PDase and AP

These observations suggest that osmotic stress, with 2 M or 3 M NaCl under low phosphate conditions, stimulates further production of PC PCH and thereby *P fellutanum* recruits more phosphocholine from extracellular pPGMs This process promotes intracellular accumulation of glycine betaine and choline-O-sulfate and thereby enhances osmotolerance of this fungus Thus it appears that extracellular phosphocholine-containing pPGM and PC PCH activity are involved in osmoregulation, as well as storage of choline derivatives, in *P fellutanum*

The data suggests that there may be a relationship between the phosphate and osmotic stress regulons in *Penicillium fellutanum*, although more direct evidence is needed to support this suggestion. In recent reports, interesting connections are beginning to be revealed between the phosphate and osmotic stress regulons in bacteria (Meyer et al., 1990; D'souza-Ault et al., 1993; Kaenjak et al., 1993). In *Pseudomonas aeruginosa*, virulence factor phospholipase C is inducible by low phosphate concentrations, but induction is inhibited by high NaCl concentrations (Shortridge et al., 1992). However, the inhibitory effect of NaCl can be overcome by the osmoprotectants glycine betaine and choline. Phospholipase C is induced by choline and glycine betaine independent of phosphate concentration. Phospholipase C is believed to function in scavenging phosphate by producing phosphocholine from phosphatidylcholine, with phosphocholine being cleaved by alkaline phosphatase. In *Staphylococcus aureus*, which grows in media with up to 3.5 M NaCl, choline, glycine betaine, and L-proline are the major osmoprotectants (Graham and Wilkinson, 1992). Large amount of glycine betaine, derived from exogenous choline, accumulated inside of the cell when *S. aureus* was grown in high salt medium (Kaenjak et al., 1993). Transport of choline into the cell is induced by osmotic stress and low phosphate concentrations; it is repressed by high phosphate concentrations (Kaenjak et al., 1993).

^{13}C NMR Spectrometry in the Study of Osmoregulation

^{13}C-NMR spectrometry is a powerful technique for studying the role of organic solutes in osmoregulation, as all the major organic solutes in intact cells and in cell extracts can be identified and quantified (Norton, 1980). Although the technique is relatively insensitive compared with ^{1}H-NMR, and the natural abundance of ^{13}C is only 1.1%, solutes that are important in osmoregulation are generally present in sufficient concentrations to allow good spectra to be obtained in a relatively short time (Borowitzka et al., 1980; Reed et al., 1984).

Many osmoregulatory compatible solutes in fungi including yeasts have been identified and quantified by ^{13}C-NMR spectroscopy (Blomberg and Adler, 1992). In marine mollusc *Tapes watlingi*, betaine, taurine, and glycine were detected by ^{13}C-NMR as major osmoregulatory solutes (Norton, 1979; Norton and de Rome, 1980). Glucosylglycerol, which is known as the major osmoregulatory solute in all species of marine cyanobacteria (Mackay et al., 1983), was initially found by this technique in a cyanobacterium, *Synechococcus* sp. (Borowitzka et al., 1980). Analysis by NMR of yeasts, *Saccharomyces cerevisiae*, *Zygosaccharomyces rouxii*, and *Debaryomyces hansenii*, grown in media containing 0 to 0.86 M NaCl showed that glycerol was the major osmoresponsive organic solute (Reed et al., 1987). In *Hansenullar anomala*, glycerol, arabinitol, and trehalose were detected by ^{13}C NMR spectroscopy as the main internal organic solutes (Belfinger and Larher, 1988). A new compatible solute, di-myo-inositol-phosphate was detected and identified in the order *Thermotogales*, which are hyperthermophilic bacteria, by NMR spectroscopy (Martins et al., 1996). Many compatible solutes such as quaternary ammonium and tertiary sulfonium compounds and amino acid derivatives in marine algae (Blunden and Gordon, 1986) and in higher plants (Rhodes and Hanson, 1993) have been characterised by ^{1}H-NMR spectroscopy.

To my knowledge, choline as a precursor of an osmoprotectant in *Penicillium* and *Aspergillus* species including yeasts has not been described before. Furthermore, this is the first demonstration of glycine betaine and choline-O-sulfate as osmoregulatory solutes in *Penicillium* species. One of the reason that these choline derivatives have not been reported before as osmoregulatory solutes in *Penicillium* and other fungi is probably due to the accumulation of high level of polyols, which is a common phenomenon to nearly all species of fungi including yeasts except Oomycetes. Another explanation relates to the complexity and insensitivity of techniques for analysis of intracellular solutes.

Throughout this study, ^{13}C NMR spectrometry was used for the detection, identification, and quantification of intracellular solutes. The metabolic labeling of intracellular solutes (especially methyl-carbon containing solutes) with stable isotope by adding either L-[*methyl*-^{13}C]methionine or [2-^{13}C]glycine (described in Chapter 3) was particularly useful in determining the osmoregulatory role of glycine betaine and choline-O-sulfate, even under the high level accumulation of polyols in the same mycelia.

CHAPTER 6
CONCLUSION

Penicillium fellutanum produces mainly two classes of exocellular complex phosphorylated peptido-polysaccharides: a soluble extracellular peptidophosphogalactomannan (pPGM) and a membrane-bound lipo-peptidophosphogalactomannan (lipo-pPGM) (Preston et al., 1969; Rick et al., 1974; Beachy, 1977; Gander et al., 1980; Gander and Laybourn, 1981). The major objectives of this research were to determine the physiological role(s) of phosphodiester residues, especially α-D-mannosyl-phosphocholine residues attached to extracellular peptidophosphogalactomannan (pPGM) of *Penicillium fellutanum*.

A C-13 methyl labeled pPGM ([*methyl*-^{13}C]pPGM) was prepared by supplementing the culture with L-[*methyl*-^{13}C]methionine and used as a probe to monitor the movement of phosphocholine residues in extracellular pPGM. It was shown, using mainly ^{13}C-NMR spectrometry, that the mycelia accumulate two major intracellular choline derivatives and that these choline derivatives originate from the phosphocholine residues in extracellular pPGM. The two intracellular choline derivatives were identified as glycine betaine and choline-O-sulfate, respectively. This suggested that extracellular pPGM is an extracellular reserve of choline in *P. fellutanum*.

The accumulation of choline-O-sulfate and glycine betaine was more significant in the mycelia grown in media containing 2 mM phosphate than in media containing 20 mM phosphate The level of intracellular glycine and choline-O-sulfate correlates with the high level production of a nonspecific phosphocholine diester phosphocholine hydrolase (PC PCH) under low phosphate conditions

The accumulation of choline O-sulfate and glycine betaine was dependent on the availability of sulfate in the low phosphate culture medium, under sulfate sufficient conditions, the level of choline-O-sulfate increased with concomitant decreased level of glycine betaine and, upon sulfate deficiency the level of glycine betaine increased with decreased level of choline-O-sulfate Free choline was not detected in significant level in the *P fellutanum* mycelia even under a sulfate deficiency The results suggest that glycine betaine may be another endogenous reserve form of choline in the mycelia growing under low phosphate conditions

Penicillium fellutanum grows in the medium stressed with up to 3 M NaCl, indicating that it is an osmotolerant and xerotolerant fungus Thus, a series of experiments was performed to determine if the phosphocholine residues in extracellular pPGM, and the two intracellular choline derivatives, are involved in adaptation processes of *P fellutanum* to environmental osmotic stress

Accumulation of glycerol and glycine betaine significantly increased in the mycelia grown for 8 days in a low phosphate medium stressed with over 1 M NaCl, however, their levels significantly decreased within 3 days due to osmotic down-shock. This result indicated that glycerol and glycine betaine are the osmoregulatory compatible solutes in *P fellutanum* grown under low phosphate and high osmolarity conditions The accumulation of choline-O-sulfate upon salt stress was less significant comparing with that of glycine betaine However, it should be noted that accumulation of choline-O-sulfate is dependent on the availability of sulfate, suggesting that its role as an osmoregulatory solute would become more significant with enough sulfate. Erythritol was also an osmoregulatory solute, although it accumulates in the mycelia stressed with only up to 2 M NaCl; at 3 M NaCl, glycerol replaces erythritol. The accumulation of glycine betaine and choline-O-sulfate occurs in the mycelia grown in low phosphate conditions even without salt stress and their levels significantly increase due to stress of 2 M or greater amounts of NaCl, indicating that they are constitutive compatible solutes. The exogenously added choline chloride (2 mM) conferred enhanced osmotolerance on *P. fellutanum* grown in the low phosphate medium stressed with 2 or 3 M NaCl. Exogenously added sulfate (10 mM) also enhanced osmotolerance of this fungus against 2 M NaCl stress. To my knowledge glycine betaine (and choline) and choline-O-sulfate have not been reported as osmoregulatory compatible solutes in filamentous fungi including *Penicillium* and *Aspergillus* species and yeasts.

The production of PC PCH was not significantly inhibited through salt stress up to 3 M NaCl, while that of other two phosphoesterases (AP and Bis-PDase), which are induced together with PC PCH under low phosphate conditions, was significantly inhibited with over 1 M NaCl stress. Furthermore, PC PCH was produced for a longer period of time in the cultures stressed with over 1 M NaCl when compared with the cultures stressed with up to 1 M NaCl. This demonstrates a certain correlation between the high osmotic stress (2M or greater amounts of NaCl) and the activity of PC PCH, which releases phosphocholine from extracellular pPGM. It was also shown that, with 0.5 M NaCl stress, the mycelia grown on low phosphate agar plates showed less growth than those grown on high phosphate plates, however, with over 2 M NaCl stress, the former showed more growth than the latter. Thus indicated that the mycelia grown under low phosphate condition in the culture medium, a condition which produces high level of PC PCH and thus recruits more phosphocholine from extracellular pPGM, show an enhanced level of osmotolerance compared to the mycelia grown under high phosphate conditions. The ^{31}P-NMR spectra of the 3 M NaCl-stressed pPGMs showed that phosphocholine residues are preferentially released as shown by comparing the phosphocholine signal with that of other phosphodiester residues in the same pPGMs. The mycelia grown under the same conditions showed increased accumulation of glycine betaine and choline-O-sulfate.

These results considered together, indicate that phosphocholine in extracellular peptidophosphogalactomannan (pPGM) with the high level production of PC PCH, is involved in osmoregulation in *P. fellutanum* grown under low phosphate and high osmolarity (2 M or greater amounts of NaCl) conditions.

It seems that the high proliferation and adaptability of fungi to their changing environments is aided at least partly by producing various extracellular hydrolases and other enzymes and then by depolymerizing or modulating the compositions of this class of extracellular and cell wall complex polysaccharides, thereby providing additional nutrients or components for cells to manage and survive their environmental stresses.

APPENDIX A
MEDIUM COMPOSITION

Table 6. Czapek-Dox Agar

Nutrients	Constituents	Concentration mM	Concentration g/l
Carbon	Glucose	278	50
Nitrogen/Sodium	$NaNO_3$	23.5	2
Phosphorus/Potassium	KH_2PO_4	7.4	1
Sulfur/Magnesium	$MgSO_4 \cdot 7H_2O$	4.1	1
Potassium	KCl	6.7	0.5
Sulfur/Iron	$FeSO_4 \cdot 7H_2O$	0.06×10^{-3}	0.018
Agar	--	--	20

Glucose was dissolved in ddH_2O, other constituents were dissolved in tap water. Micronutrients were added as described in Table 6. After autoclaving, the above were mixed and dispensed into each sterile plastic dish as soon as the flasks could be handled.

Table 7 Standard Growth Medium (SG)

Nutrients	Constituents	Concentration	
Macronutrients		mM	g/L
Carbon	Glucose	320	57.6
Nitrogen	NH_4Cl	80	4.3
Phosphorus	Na_2HPO_4	20	2.8
Sulfur	Na_2SO_4	2	0.28
Buffer/Chelator	Na citrate	12.5	3
	Citric acid	12.5	2.2
Potassium	K_2CO_3	3	0.42
Magnesium	$MgCO_3$	4	0.34
Sodium/Chloride	NaCl	20	1.2
Micronutrients		ppm	mg/L
Iron	$FeSO_4 \cdot 7H_2O$	10	50
Zinc	$ZnCl_2$	5	10.4
Manganese	$MnCl_2 \cdot 4H_2O$	5	18.0
Molybdenum	$(NH_4)_6(Mo_7O24) \cdot 4H_2O$	2	3.7
Calcium	$CaCl_2 \cdot 5H_2O$	0.5	1.8
Copper	$CuSO_4 \cdot 5H_2O$	0.4	1.6
Vanadium	NH_4VO_3	0.2	0.46
Boron	H_3BO_3	0.1	0.57
Chromium	$Cr_2(SO_4)_3 \cdot 12H_2O$	0.1	0.93
Nickel	$NiCl_2 \cdot 6H_2O$	0.1	0.4
Cobalt	$CoCl_2 \cdot 6H_2O$	0.1	0.4

Micronutrients, except for $FeSO_4$, were stored as individual 4,000-fold concentrated stock solutions. Before autoclaving each medium, 0.25 ml/L final volume of each and solid $FeSO_4$ were added to the macronutrient solution. Carbon source and combined salts were separately dissolved in ddH_2O, autoclaved at 121°C for 25 min, and combined aseptically after cooling. The pH of combined medium was 5.95. Overall carbon/nitrogen molar ratio equals 26.

APPENDIX B
CHARACTERIZATION OF PEPTIDOPHOSPHOGALACTOMANNANS (pPGMs) PRELIMINARY DATA

To determine whether high salt concentrations in the culture medium caused compositional changes in extracellular peptidophosphogalactomannans (pPGM) during cell lysis, the compositions of pPGMs isolated from high phosphate culture medium (SG) supplemented with 5 M NaCl were analyzed and compared with those of unstressed pPGMs

Total Carbohydrate

Total carbohydrate of each pPGM species was determined by the phenol-sulfuric acid method of Dubois et al (1956) Each pPGM sample was dissolved in ddH_2O Samples in a total volume of 0 3 ml solution were taken and mixed thoroughly after addition of 20 µl of 80% phenol and 1 0 ml of concentrated H_2SO_4. The absorbance values determined at 490 nm were compared with a reference solution of 0 242 mM glucose

Total Phosphate

Total phosphate was determined by the ashing technique of Ames and Dubin (1960) and the color reagent of Ames (1966) Each pPGM sample was dissolved in

ddH_2O. Aliquots of samples (20-100 µl) in glass test tubes were taken to dryness over an open flame after addition of 60 µl of 10% $Mg(NO_3)_2 \cdot 6H_2O$. To avoid contamination, the test tubes used were pre-treated with concentrated HCl and thoroughly rinsed with ddH_2O. The heating was continued until brown fumes completely disappeared. The dried residue were then treated with 0.6 ml of 0.5 N HCl and the mixture was placed in a boiling water bath for 15 min. to hydrolyze any pyrophosphate. After cooling, 1.4 ml of a mixture of molybdenum-ascorbate reagent (0.42% ammonium molybdate in 1 N H_2SO_4 : 10% ascorbic acid in ddH_2O, 6:1, v/v) was added and the final solution was incubated at 45°C for 20 min. The absorbances were then determined at 820 nm and the values obtained were compared with a reference solution of 0.4 mM KH_2PO_4.

Amino Acid Analysis

Five milligrams of each sample of pPGM in a tightly capped glass bottle was hydrolyzed in 1 ml of 6 N HCl at 100°C for 4 h. After cooling, the hydrolysate was transferred to 18 x 150 mm pyrex tubes and evaporated in vacuo at 35°C to dryness. Evaporation was repeated 4 ~ 5 times to completely remove residual HCl. The sample was dissolved in 40 µl of ddH_2O and spotted on the Whatman No. 3 paper. The reference amino acids were Lys, Leu, Glu, Pro, Val, Gly, Ala, Thr and Ser (1.5 mg of each amino acid dissolved in 1 ml of ddH_2O). Ten microliters (15 µg) of each amino acid solution was spotted. The chromatogram was developed in a descending manner for 24 h with *n*-butanol:pyridine:ddH_2O (6:4:3, v/v/v). The chromatogram was then air dried for 4 h and the spots were visualyzed by spraying 2% ninhydrin-acetone solution and heating at 100°C for 5 min.

Carbohydrate Composition

Five milligrams of each pPGM was hydrolyzed in 0.5 ml 2 N HCl at 100°C for 2 h and then evaporated *in vacuo*, dissolved in 0.5 ml ddH_2O and neutralyzed with 0.5 ml 2 N NaOH. Ten microliter of acid hydrolyzed sample was spotted on Whatman No. 3 paper and developed in a descending manner with n-butanol:pyridine:ddH_2O (6:4:3, v/v/v) for 14 h at room temperature in a glass tank lined with the same paper previously saturated with the solvent employed. Ten microliter of each reference carbohydrate (5 mM glucose, 5 mM galactose, 5 mM mannose, and 20 mM erythritol) was spotted. The bottom edge of the chromatogram was serrated, and the solvent was allowed to drip off the chromatogram. The chromatogram was then taken out of the developing tank, dried and soaked into the first staining solution (mixture of 2 ml of 40% $AgNO_3$ and 80 ml of acetone). After air drying, it was treated with a mixture of 10 ml of 10 N NaOH and 90 ml of ethanol. The wet chromatogram was finally heat dried for 5 min in 100°C drying oven, fixed with 0.2 M $Na_2S_2O_3$, and then air dried in the hood.

Optical Rotation

Optical rotation was determined on 4 mg of each pPGM sample dissolved in 1.5 ml ddH_2O in a one decimeter cuvette using a Jasco DIP Digital polarimeter. Integration time was 5 sec.

Molecular Weight

Molecular weight of each pPGM species was determined by Bio-gel P-30 gel-permeation chromatography using reference proteins as M.W. markers (Bio-Rad) thyroglobulin, 670 kDa, gamma globulin, 158 kDa, ovalbumin, 44 kDa, myoglobin, 17 kDa, vitamin B-12, 1,350 Da. The chromatography was performed at room temperature. To reduce the wall effect, the column (76 x 1.7 cm) was silanized with 5% solution of dimethyldichlorosilane in toluene before packing the gels. After packing, the column was preequilibrated with a buffer (10 mM Tris-acetate buffer, pH 7.0, containing 0.1 M KCl and 0.1 M NaCl). Ten milligrams of pPGM sample dissolved in 0.3 ml of the same buffer was applied to the column and eluted at a flow rate of 11.2 ml per 1 h with the fraction volume of 820 µl. Blue Dextran 2 000 (1 mg) was used for the determination of Vo. The fraction was assayed for total carbohydrate by phenol-sulfuric acid method at 490 nm and for proteins at 280 nm.

Determination of O-Glycosidic Linkage by α-, β-elimination

The presence of O-glycosidic linkage between reducing end of mannan backbone and 3 kDa peptide (Rick et al., 1974) in each pPGM species was determined by α- and β-elimination reactions. Four milligrams of each pPGM was dissolved in 0.9 ml of ddH$_2$O and transferred to a 1 ml cuvette and to this 0.1 ml of 1 N NaOH was added. The absorbance change was monitored during incubation for ~ 4 h at 241 nm using a Hewlett Packard Model A Diode Array spectrophotometer.

Results

The compositions of the salt-stressed pPGM obtained during cell lysis in high phosphate SG medium stressed with 5 M NaCl for 13 days were compared with those of the normal pPGM. Only 34% by weight of pPGM was obtained from 5 M NaCl stressed medium when compared with the pPGM from control SG medium (Table 8). The salt-stressed pPGM showed a ratio of total carbohydrate to total phosphate of about 2.18-fold greater than that of normal pPGM, the ratios of total carbohydrate to total phosphate of normal pPGM and salt stresed-pPGM were 12.8/1 and 28/1, respectively. Like normal pPGM, it also contained O-glycosidic linkages where mannose and mannooligosaccharides are attached to serine or threonine residues in the 3 kDa peptide. However, it showed positive values in optical rotation, indicating that it contained very little or no galactofuranosyl residues. This conclusion was confirmed by demonstrating that the only sugar in acid hydrolysates of the sample was mannose. Paper chromatography of 6 N acid hydrolysates of the sample showed only one major ninhydrin-positive spot that matched with Ser. The peptide did not contain threonine, alanine, lysine or proline, which are major amino acid species that occur in the 3 kDa peptide in normal pPGM. Peptide of normal pPGM contains about 50% Ser and Thr residues (Rick et al., 1974). The mass of salt stressed pPGM is about 35 kDa as estimated by gel-permeation chromatography on Bio-Gel P-30; that of normal pPGM is about 65 kDa.

Table 8 Characterization and comparison of peptidophosphogalactomannans obtained from NaCl-stressed cultures

	pPGM[1]	pPGM[2]
Total amount of CHO (mg/1 6 L)[3]	320 (100%)	110 (34%)
Total PO$_4$/CHO[4]	1/12 8	1/28
Optical rotation[5] (in degrees)	- 0 153	0 001
O-glycosidic linkage[6]	yes	yes
Galactose[7]	yes	ND
Mannose[7]	yes	yes
Amino acid contents[8]	Ser Thr, Ala, Pro Lys, Gly, Val, Asp Asn, Glu, Gln, His	Ser[9]
M.W [10]	65 kDa	35 kDa

[1] Normal pPGM from SG medium with 20 mM NaCl [2] pPGM from SG medium with 5 M NaCl [3] Total carbohydrate by weight from 1 6 L culture filtrate [4] Total PO$_4$ estimated after Mg(NO$_3$)$_2$ oxidation of pPGM Total carbohydrate (CHO) estimated by phenol-H$_2$SO$_4$ method [5] Optical rotation with 4 mg of pPGM in 1 5 ml ddH$_2$O Integration time was 5 sec [6] Determined by α- β-elimination with 4 mg pPGM in 0 1 N NaOH at 241 nm for 4 h. Hydrolysis of 5 mg pPGM in 0 5 ml 2 N HCl at 100°C for 2 h, rotary evaporated, dissolved in 0 5 ml ddH$_2$O and neutralized with 0 5 ml 2 N NaOH. Acid hydrolyzed sample (10 µl) was spotted on whatman No 3 paper, eluted with 50 ml of n-butanol pyridine ddH$_2$O (6 4 3 v/v/v) for 14 h, and stained with 40% AgNO$_3$ in 0 3 M Na$_2$S$_2$O$_3$ [8] Hydrolysis of 5 mg pPGM in 1 0 ml of 6 N HCl at 100°C for 4 h, and chromatographed on paper, developed by 2% ninhydrin. [9] Molecular weight determined by Bio-Gel P 30 gel-permeation chromatography Symbols yes, detected, ND, not detected.

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BIOGRAPHICAL SKETCH

Yong-Il Park was born in South Korea on July 24 1961 He graduated from Chun-Cheon High School in February 1980 Yong Il entered the school of engineering, Kon Kuk University in Seoul, where he specialized in Microbial Engineering After 2 years of college study, he was drafted into the military camp in June, 1982 He was discharged from the service in April, 1984 and returned to the college In February, 1987 he graduated with a Bachelor of Engineering degree from the Department of Microbial Engineering at Kon-Kuk University During university years, Yong-Il joined Prof Chung's laboratory as an undergraduate student and continued working with Dr Chung through his graduate study In 1989, Yong-Il received a Master of Science degree from the Graduate School of Kon-Kuk University with a major in fermentation technology He continued working as a teaching assistant in the same department After studying for 1 year as a master's student in the Microbiology and Immunology department at McGill University in Montreal, Canada, he began his graduate study towards attaining his doctorate in the Department of Microbiology and Cell Science at University of Florida in 1992 Since 1992, he has been working with Dr John E Gander who has introduced him to the wonderful worlds of fungal polysaccharides, enzymology and nuclear magnetic resonance (NMR) spectrometry

He is married to Young-Nan and has a three-year-old daughter, Hye-Seun.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy

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